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Metabolic Engineering of a heterologous CO2-fixation in Saccharomyces cerevisiae

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Zusammenfassung

Eine der größten Herausforderungen des 21. Jahrhunderts ist die steigende Nachfrage nach Energie für Transport, Heizung und industrielle Prozesse zu decken. Aufgrund der Sorge um die Sicherheit der Ölversorgung und der negativen Auswirkungen der Nutzung fossiler Brennstoffe auf die Umwelt steigt das weltweite Interesse nach der Erschließung alternativer, nachhaltiger Energiequellen. Der derzeit wichtigste Biokraftstoff ist Ethanol. Ein Großteil des weltweit produzierten Bioethanols wird durch mikrobielle Fermentation von Biomasse gewonnen. Die Bäckerhefe ist dabei für gewöhnlich die erste Wahl für die industrielle Produktion. Die Herstellung und Verwendung von Bioethanol aus zucker- und stärkehaltigen Materialien ermöglicht eine Reduktion der weltweiten Erdölabhängigkeit und eine Reduktion der Abgasemissionen und ist daher aus ökologischer und ökonomischer Sicht von außerordentlichem Interesse.

Bei der mikrobiellen Ethanol Gewinnung wird typischerweise Ethanol und CO_2 zu gleichen Massenteilen aus Glukose gewonnen. Ein Drittel des wertvollen Zucker-Kohlenstoffs geht daher als eines der wichtigsten Treibhausgase CO_2 verloren. Eine kombinierte Verstoffwechslung von Glukose und CO_2 in Form einer Totalbiosynthese zu Ethanol wäre aus ökonomischen (mehr Ethanol pro Kulturpflanze) aber auch aus ökologischen (CO_2-freier Prozess) Gründen sehr interessant. Leider ist kein mikrobieller Ethanol Produzent bekannt der das entstehende CO_2 nutzbringend wiederverwerten kann und jene (Mikro)Organsimen die dazu in der Lage sind, sind nicht für die Fermentation von Ethanol aus Glukose programmiert. Hingegen können bestimmte Bakterien wie *Moorella thermoacetica* das bei der Fermentation von Glukose anfallende CO_2 quantitativ in Form von Essigsäure wieder verwerten. Die zugrundeliegende CO_2-Fixierung kann aus thermodynamischen und stöchiometrischen Überlegungen mit dem Ethanol Stoffwechsel der Bäckerhefe kombiniert werden und soll eine Ertragssteigerung um 50 % ermöglichen. Ausgehend von dieser Idee wurden im Zuge der Masterarbeit rekombinante Bäckerhefestämme konstruiert, die jene Enzyme und Proteine die an der Fixierung von CO_2 in *M. thermoacetica* beteiligt sind, heterolog exprimieren. Dazu wurde eine geeignete Klonierungsstrategie entwickelt und umgesetzt. Erhaltene Stammkonstrukte wurden hinsichtlich korrekter chromosomaler Integration der Zielgene durch diagnostische PCR geprüft. Die Transkription der Gene wurde mit qPCR verifiziert und die funktionale Expression der rekombinanten Proteine und Enzyme mit geeigneten Testverfahren geprüft.

Abstract

One of the biggest challenges of the 21st century is to meet the growing energy demand for transport, heating and industrial processes. Due to the concern for the security of oil supply and the negative impact of fossil fuels on the environment, worldwide interest rises for the development of alternative and sustainable energy sources. Currently the main biofuel is ethanol. Most bioethanol worldwide produced is obtained by microbial fermentation of biomass. The baker's yeast is usually the first choice for industrial production. The production and use of bioethanol from sugar- and starch- based feedstock enables a reduction in global oil dependency and emission reduction and is therefore from an ecological and economic point of view of extraordinary interest. In the microbial ethanol production typically ethanol and CO_2 are formed at equal mass parts from glucose. One third of the valuable sugar-carbon is thus lost as one of the most important greenhouse gas CO_2. A combined utilization of glucose and CO_2 to ethanol, in the form of a total biosynthesis, would be for economic reasons (more ethanol per crop) but also for environmental reasons (CO_2-free process) very interesting. Unfortunately, no microbial ethanol producer is known that can recycle the resulting CO_2 useful and those microorganisms that are capable, are not programmed for the fermentation of ethanol from glucose. On the other hand, certain bacteria such as *Moorella thermoacetica* can quantitatively recycle the CO_2 produced during glucose fermentation in the form of acetic acid. The underlying CO_2 fixation can be, based on thermodynamic and stoichiometric considerations, combined with the ethanol metabolism of baker's yeast and should enable a 50 % increase in yield. Based on this idea recombinant baker's yeast strains were constructed, heterologously expressing the proteins and enzymes involved in the fixation of CO_2 in *M. thermoacetica*. For this purpose, a suitable cloning strategy was developed and implemented. Strain constructs obtained were tested for proper chromosomal integration of the target genes by diagnostic PCR. The transcription of the genes was verified using qPCR and subsequently, the functional expression of the recombinant proteins and enzymes were examined with suitable enzyme assays.

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List of Abbreviations

EMP	Embden-Meyerhof pathway
ADH	Alcohol dehydrogenase
mTHF	Methyl-tetrahydrofolate
CoA	Coenzyme A
acetyl-CoA	Acetyl Coenzym A
mTR	Methyltransferase
CFeSP	Corrinoid/iron-sulfur protein
CODH	Carbon monoxide dehydrogenase
Fd	Ferredoxin
ACS	Acetyl-CoA synthase
THD	Transhydrogenase
PFOR	Pyruvate-ferredoxin oxidoreductase
ACDH	Acetaldehyde dehydrogenase
BSA	Bovine serum albumin
DTT	Dithiothreitol
YPD	Yeast Peptone Dextrose Medium
LB	Luria-Bertani Medium
ONCs	Overnight cultures
ICH_3	Iodomethane
(v/v)	Volume/Volume
(w/v)	Mass/Volume
OD_{600}	Optical density at 600 nm wavelength
ntc	Non-template control
NTR	No reverse transcriptase control
Ct	Cycle threshold
WT	Wild type
LC-MS	Liquid chromatography coupled with mass spectrometry
rpm	Rounds per minute
NAD(P)H	β-Nicotinamide adenine dinucleotide (phosphate) - reduced form
NAD(P)+	β-Nicotinamide adenine dinucleotide (phosphate) - oxidized form
μ	Specific growth rate
Tm	Melting temperature

Contents

1 Introduction

1.1 Increasing global demand for energy

Global energy and therefore oil and gas demands are steadily increasing due to various factors including the growing world population, increasing wealth in emerging markets such as India and China and increased transportation as a result of globalization and strong economic growth. It is estimated that the worldwide oil demand is about to rise around 1 % per year [1], with transportation as the main consumption sectors. The main share of the liquid fuel used is derived from petroleum [2]. But as its use is estimated to be the main contributor to total greenhouse gas emission and because the prices for crude oil are increasing as the petroleum resources are prospected to decrease, worldwide interest in developing renewable, sustainable and carbon dioxide neutral alternatives for fossil fuels rise [3].

1.2 Renewable fuels for transportation

With the emerging environmental and security concerns of future energy supply renewable energy in form of biofuels such as bioethanol, biodiesel, and biogas is becoming more and more interesting to supply transportation fuel demand. Biofuels which are defined as liquid or gaseous fuels can be derived from biomass such as sugar- and starch-based ethanol, from oil-crop like biodiesel, or from anaerobic digestion like biogas [4]. Their production with microorganisms from renewable feedstock is just one of several promising alternatives.
Because renewable fuels such as bioethanol can be used directly as fuels or fuel additives for already existing motor vehicles, without the need for significant motor adaptations, they offer a promising way for the reduction of CO_2 emission [5]. Some countries have already acknowledged these advantages and have initiated directives in order to replace fossil fuels. The US Energy Policy Act of 2005 for example stated that the oil industry is required to blend 33,75 billion liter of renewable fuels into gasoline by 2012 [6]. Moreover the US Department of Energy has set a goal of replacing 30 % of transportation fuels with bioethanol and biodiesel by 2025 while in the European Union through Directive (EU) 2015/1513 each Member State is required to ensure that by 2020 10 % of the energy used for transportation is drawn from renewable sources [7, 8].

1.3 Bioethanol

Currently the most commonly used renewable fuel for transportation is bioethanol [9]. It is one of the most promising alternatives and is mainly derived from corn grain (starch) and sugar cane (sucrose) [6]. For its production food crops as well as waste feedstock can be used which are both abundant and renewable [6]. Bioethanol can be used for transportation directly or in blends with gasoline with a share of 10 to 85 % [10]. Derived from corn grain it yields 25 % more energy than is required for its production [11] and its use as fossil fuel substitute is estimated to reduce greenhouse gas emission up to 12 % [11].
However, there are some disadvantages of the bioethanol production from food crops such as its high production costs due to high feedstock prices, the direct competition of the used raw material with its use as staple food, increased water demand and arable land shortage [12]. Nevertheless still most of the bioethanol is produced by fermentation of feedstock such as corn and sugar beet [13]. To continue permitting biofuels such as bioethanol to compete with petroleum, attempts for developing more efficient processes for bioethanol production are from ecological and economic interest.

1.3.1 Ethanol production organism

Various microorganisms including *S. cerevisiae*, *Zymomonas mobilis* and recombinant *E. coli* strains are capable of producing bioethanol from glucose [14, 15]. Owing to its high ethanol tolerance, good fermentative capacity and fast growth under anaerobic conditions baker's yeast (*S. cerevisiae*) is the microorganism used most intensively for bioethanol production [9]. It offers many advantages with regard to metabolic engineering as it can handle harsh industrial conditions like tolerating a wide range of pH (which can reduce contaminations) [16], its well-studied physiology and metabolism and the availability of tools for genetic engineering [17].

1.4 Biochemistry of alcohol fermentation in *Saccharomyces cerevisiae*

The main substrate used for ethanol production with baker's yeast is glucose. The first step in ethanol production is the glucose metabolization via the Embden-Meyerhof pathway (EMP) to pyruvate.

Net Reaction Equation: Glucose + 2 P_i + 2 ADP + 2 NAD^+ = 2 pyruvate + 2 ATP + 2 NADH + 2 H^+ + 2 H_2O

Under anaerobic conditions, in a process known as alcoholic fermentation, the pyruvate decarboxylase and alcohol dehydrogenase (ADH) then catalyze the conversion of pyruvate into ethanol and CO_2 [18].

Net Reaction Equation: Glucose + 2 P_i + 2 ADP + 2 H^+ = 2 ethanol + 2 CO_2 + 2 ATP + $2H_2O$

1.4.1 Strategies to improve bioethanol production in *S. cerevisiae*

To meet the demand of the steadily growing bioethanol market, strategies have been established for a more cost effective ethanol production from biomass. A lot of effort is put into the introduction of novel pathways or optimization of cellular processes. Approaches include increasing the yeasts ethanol tolerance [19], metabolic engineering of biofuel crops to increase biomass production or the sugar/starch content per kg plant [19, 20, 21] or redirecting the carbon flow from biomass, glycerol and CO_2, which are the main products of glucose conversion under anaerobic conditions besides ethanol [22, 23] (see Figure 1.1) towards ethanol biosynthesis to increase the ethanol conversion efficiency.

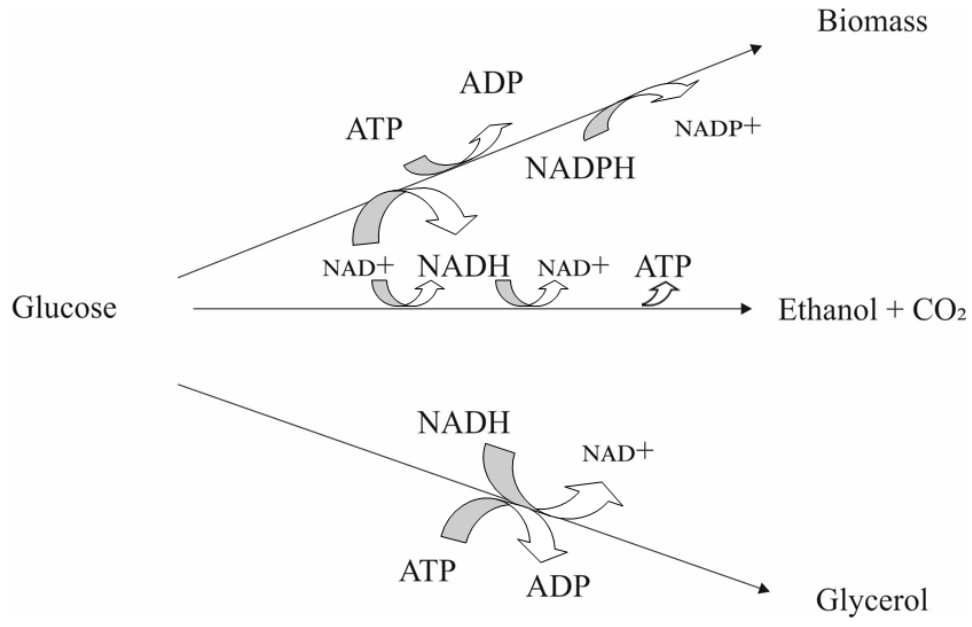


Figure 1.1: Main carbon flow and its cofactor requirement in *S. cerevisiae* grown anaerobic on glucose [23].

Redirecting the carbon flow from biomass production towards ethanol biosynthesis can be achieved by increasing the ATP consumption during biomass formation [24], while engineering the redox metabolism in *S. cerevisiae* represents a possibility to redirect carbon flow from glycerol towards ethanol synthesis [23]. Expressing a non-phosphorylating, $NADP^+$ dependent glyceraldehyde-3-phosphate dehydrogenase under anaerobic conditions on glucose has also shown to be capable of reducing glycerol by-product formation in *S. cerevisiae* [23, 25, 26].

Redirecting CO_2 towards ethanol synthesis represents another very interesting strategy to avoid loss of the valuable carbon equivalent as one of the most important greenhouse gases CO_2 and to increase the overall carbon yield.

1.5 CO_2 fixation pathways

While in all plants and many prokaryotes the predominant way of CO_2 fixation into cellular carbon is via the reductive pentose phosphate cycle, other prokaryotes (listed in Table 1.1) have developed different autotrophic pathways for CO_2 recycling.

Table1.1: CO_2 fixation pathways. Different pathways evolved for the fixation of CO_2 in different organism.

CO_2 fixation pathway	Microorganisms	References
- Reductive citric acid cycle (Arnon-Buchanan cycle)	green sulfur bacteria and Proteobacteria (δ and ε type)	[27, 28, 29]
- 3-hydroxypropionate /4-hydroxybutyrate cycle	aerobic *Sulfolobales*	[29, 30]
- Dicarboxylate/4-hydroxybutyrate cycle	anaerobic *Thermoproteales* and *Desulfurococcales*	[30, 31]
- Hydroxypropionate (Fuchs-Holo) bi-cycle (3-Hydroxyproprionate cycle)	*Chloroflexaceae*	[4, 28, 29]
- Reductive acetyl-CoA pathway (Wood-Ljungdahl pathway)	acetogenic bacteria, methanogenic archaea, plantomycetes and sulfate-reducing bacteria	[29, 32, 33]

Unfortunately none of the so far known CO_2 recycling microorganisms is genetically programmed for the production of ethanol. Neither is *S. cerevisiae* capable of CO_2 fixation.

An interesting approach to combine CO_2 fixation and ethanol production would be the introduction of one of the above mentioned CO_2 fixation pathway in baker's yeast, enabling it to fixate the carbon dioxide and convert it into ethanol [29, 34]. Fast A. G. and Papoutsakis E.

T. recently compared the different carbon dioxide fixation pathways based on their ATP and electron requirement and the number of involved enzymes and concluded that the Wood-Ljungdahl pathway in fact is the most efficient for redirecting CO_2 towards ethanol synthesis [35].

1.6 Wood-Ljungdahl pathway

The Wood-Ljungdahl (or reductive acetyl-CoA) pathway is found in many microorganisms over a broad range of phylogenetic classes [32]. It is used for energy conservation and autotrophic carbon assimilation in acetogens, as well as for acetate catabolism by sulfate-reducing bacteria [36, 37] and aceticlastic methanogens as well as for CO_2 fixation by hydrogenotrophic methanogens [38, 39, 40, and 41].

The obligate anaerobic Clostridium *M. thermoacetica* is a model acetogen which has been used widely for studying the Wood-Ljungdahl pathway of CO and CO_2 fixation [32]. The pathway enables the *Clostridium* and other acetogenic bacteria to grow autotrophically using H_2 and CO as electron donors and CO_2 as an electron acceptor [41].

Moreover it allows almost stoichiometrically conversion of one mole glucose into three mole of acetate, which involves incorporation of two mole carbon dioxide into acetic acid. Two mole of acetate are formed via the EMP-pathway while the third mole is formed via the reductive acetyl-CoA pathway [33.

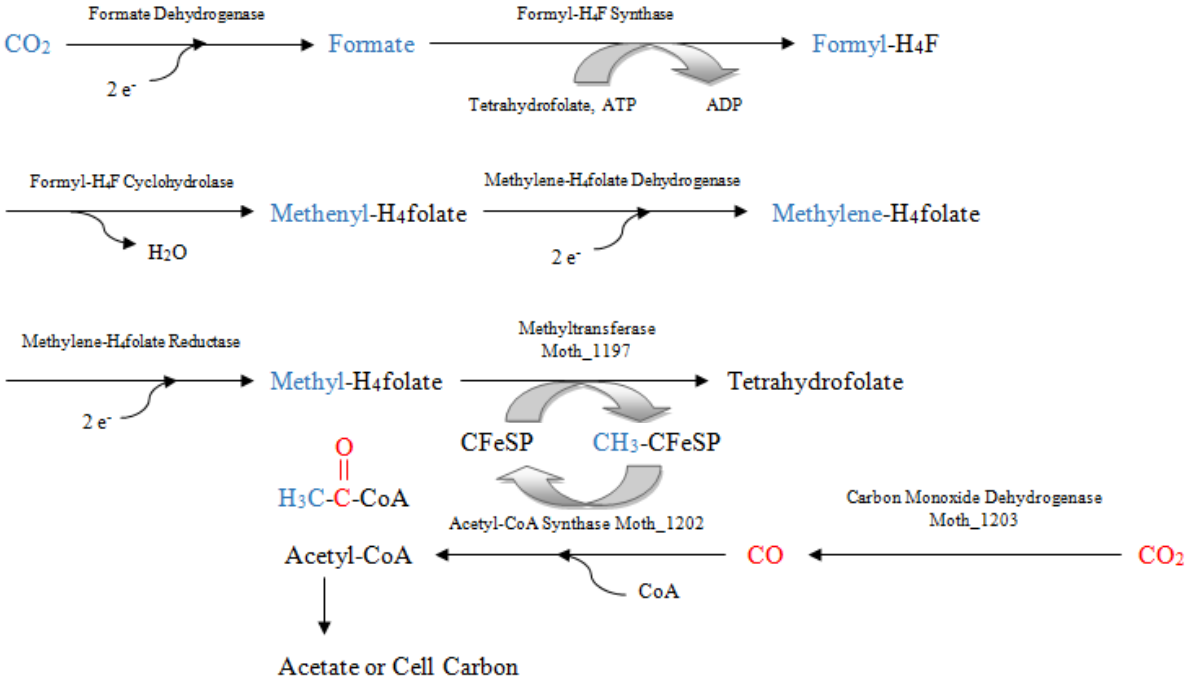


Figure 1.2: The Wood-Ljungdahl pathway of CO_2 fixation in *M. thermoacetica*. The utilization of the first CO_2 in the Eastern branch is shown in blue and the second CO_2 utilized through the Western branch is depicted in red [32].

The Wood-Ljungdahl pathway, which is shown in Figure 1.2, is divided into an Eastern and a Western branch. In the Eastern branch, can be found in many microorganisms including *S. cerevisiae*, one CO_2 is reduced to formate, which is further converted to a methyl moiety of methyl-tetrahydrofolate (mTHF). In the Western or Carbonyl branch another CO_2 is reduced to a carbonyl group, which is transferred together with the bound methyl group to coenzyme A (CoA) to form acetyl-CoA. The four proteins involved in the Western branch reaction are the methyltransferase (mTR), the corrinoid/iron-sulfur protein (CFeSP), carbon monoxide dehydrogenase (CODH), and ferredoxin (Fd) [32]. The genome sequence encoding enzymes and proteins constituting the Wood-Ljungdahl pathway in *M. thermoacetica* are known [33]. Many genes of the Eastern branch are spread over the whole genome, while the core genes enabling formation of acetyl-CoA from mTHF, CoA and CO_2, including the carbon monoxide dehydrogenase, acetyl-CoA synthase (ACS), methyltransferase as well as both subunits of a corrinoid iron–sulfur protein [41] are co-localized in the *acs* gene cluster as can be seen in Figure 1.3.

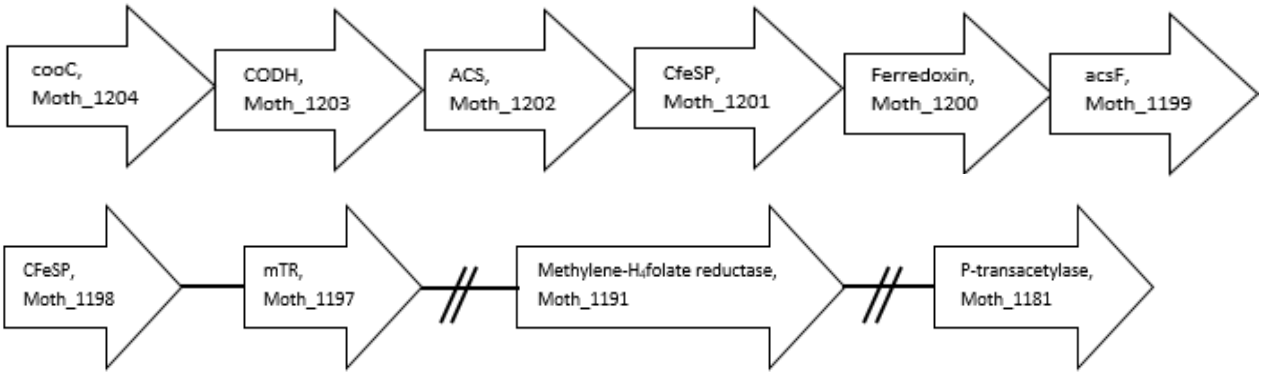


Figure 1.3: The *acs* gene cluster of *M. thermoacetica*.

1.7 Metabolic engineering of a total biosynthesis of ethanol from glucose by *S. cerevisiae*

The Western branch, in which one CO_2 is reduced to CO, is unique to acetogens, methanogens, and sulfate reducers. However based on thermodynamic and stoichiometric considerations (shown in Table 1.2 and 1.3) it could be shown that the Wood-Ljungdahl pathway is compatible with the reaction network of the central carbon metabolism of *S. cerevisiae*. Two synthetic pathway models (CAA- and CAP pathway) were suggested for the baker's yeast (M. Klimacek, unpublished data). In both models acetyl-CoA is formed from CO_2 and a methylgroup of mTHF (which is produced by *S. cerevisiae* itself from serine involving both cytosolic and mitochondrial enzymatic pathways [42]) by heterologous expression of the bifunctional enzyme carbon monoxide dehydrogenase / acetyl-CoA synthase, the methyltransferase as well as the corrinoid iron–sulfur protein from *M. thermoacetica*.

Acetyl-CoA carbon is then reintegrated into the ethanol pathway by either conversion to pyruvate catalyzed by pyruvate-ferredoxin oxidoreductase (PFOR; **CAP-pathway**) or by conversion to acetaldehyde by the acetylating acetaldehyde dehydrogenase (ACDH; **CAA-pathway**). In the CAP-pathway pyruvate is formed by recombinant PFOR utilizing CO_2, which is subsequently converted into acetaldehyde. The CAA-pathway enables direct production of acetaldehyde from acetyl-CoA by recombinant ACDH. Acetaldehyde is then further converted to ethanol in the cytoplasm or mitochondria by the NADH dependent alcohol dehydrogenase. Balancing the redox cofactors furthermore requires the presence of transhydrogenases and NADPH as well as Fd-dependent hydrogenase reactions.

The thermodynamic data showing that ethanol production from CoA, CO_2 and mTHF is thermodynamically feasible for the CAP and the CAA pathway in *S. cerevisiae* are displayed in Table 1.2 and 1.3.

Table 1.2: Model-pathway. Stoichiometry and thermodynamic data of the CAP- and CAA pathway.

CAP-pathway		
Enzyme	Reaction	$\Delta_rG'^{0a}$
CODH [EC 1.2.7.4]	$CO_2 + 2\ Fd_{red} + 2\ H^+ = CO + 2\ Fd_{ox} + H_2O$	38[b]
ACS [EC 1.2.99.2]	CO + mTHF + CoA = Acetyl-CoA + THF	-76[c]
CODH/ACS	CO_2 + mTHF + 2 Fd_{red} + CoA + 2 H^+ = AcCoA + THF + 2 Fd_{ox} + H2O	-38
	CO_2 + mTHF + H_2 + CoA = Acetyl-CoA + THF + H_2O [d]	-59
PFOR [EC 1.2.7.1]	CO_2 + Acetyl-CoA + 2 Fd_{red} + 2 H^+= pyruvate + CoA + 2 Fd_{ox}	13.7
	CO_2 + Acetyl-CoA + H_2 = pyruvate + CoA d	-7.8
PDC [EC 4.1.1.1]	pyruvate = CO_2 + acetaldehyde	-16.6
ADH [EC 1.1.1.1]	acetaldehyde + NADH + H^+= ethanol + NAD^+	-22.1
Net reaction	**CO_2 + mTHF + 2 H_2 + H^+ + NADH = ethanol + THF + NAD^+ + H_2O [d]**	-105.5
CAA-pathway		
Enzyme	Reaction	$\Delta_rG'^{0a}$
CODH/ACS	CO_2 + mTHF + H_2 + CoA = Acetyl-CoA + THF + H_2O [d]	-59
ACDH [EC 1.2.1.10]	Acetyl-CoA + NADH + H^+ = acetaldehyde + CoA + NAD^+	14
ADH [EC 1.1.1.1]	acetaldehyde + NADH + H+= ethanol + NAD^+	-22
Net reaction	**CO_2 + mTHF + H_2 + 2 H^+ + 2 NADH = ethanol + THF + 2 NAD^+ + H_2O [d]**	-67

[a] All values of $\Delta G'^0$ [kJ/mol] were calculated from transformed standard Gibbs energies $\Delta G'^0$ taken from [43], according to the underlying fundamental thermodynamic principles (Ref.: pH 7.0, 30°C). A G'^0 of -130 kJ/mol for the mTHF / THF couple was derived from [44] and respective $\Delta G'^0$s from [43]. [b] Compares well to a $\Delta G'^0$ of 37 kJ/mol calculated from midpoint potentials of -365 mV [45] and -558 mV [44] determined at pH 7.0 for $FdII_{ox}$ -> $FdII_{red}$ from *M. thermoacetica* and CO_2->CO, respectively; [c] Compares well to -65 kJ/mol determined experimentally at 37°C and at pH 8.0 [44]. [d] Fd_{red} is provided by Fd-Hyd (H_2 + 2 Fd_{ox} = 2 H+ + 2 Fd_{red}; $\Delta G'^0$ = -21.4 kJ/mol)

1.8 Recombinant expression of enzymes and proteins constituting the Western branch of the Wood-Ljungdahl pathway in *S. cerevisiae*

To summarize, linking CO_2-fixation with ethanol production in *S. cerevisiae* can, in theory, be achieved by functional expression of the CODH, ACS, CFeSP and mTR, constituting the Western branch of the Wood-Ljungdahl pathway in *M. thermoacetica*, as well as expression of recombinant PFOR or ACDH and additional transhydrogenase (THD) plus ferredoxin and NADPH hydrogenases. This undertaking is feasible under thermodynamic and stoichiometric considerations and would result in the production of 3 mole of ethanol and 1 ATP from one

mole glucose and 6 moles of H_2, enabling the microorganism to increase its ethanol yield up to 50 %.

Net reaction of glucose assimilation via the CAP pathway:

Glc + 4 H_2 + NAD^+ + ADP + P_i + 3 NADPH + 3 H^+ = 3 EtOH + ATP + NADH + 3 $NADP^+$ + 3 H_2O

Net reaction of glucose assimilation via the CAA pathway:

Glc + 2 H_2 + NADH + ADP + P_i + 3 NADPH + 5 H^+= 3 EtOH + ATP + NAD^+ + 3 $NADP^+$ + 3 H_2O

Establishing a CO_2-neutral ethanol production in *S. cerevisiae* would not only provide an environmentally friendly solution for the worldwide growing bioethanol demand, but also represent a milestone in the field of metabolic engineering.

1.8.1 Enzymology of the Western branch of the Wood-Ljungdahl pathway

The CODH, ACS, mTR, CFeSP, orf7, acsF and cooC from *M. thermoacetica* represent novel targets for the expression in *S. cerevisiae*. To gain an overview about the enzymes and proteins facilitating CO_2, CoA and mTHF conversion to acetyl-CoA, they are introduced in Table 1.4.

Table 1.4: Function and properties of enzymes and respective potential helper proteins constituting the Western branch of the Wood-Ljungdahl pathway in *M. thermoacetica*.

Enzyme/Protein name, abbreviation and EC number	Description
CFeSP methyltransferase (Moth_1197, EC 2.1.1.258)	The mTR is a B_{12}-dependent methyltransferase which catalyzes the transfer of the methyl group of methyl-tetrahydrofolate to the cobalt center of the corrinoid iron–sulfur protein [32, 33]. Reaction equation: Cob(I)amide + mTHF <-> methyl-Cob(III)amide + THF
Corrinoid iron–sulfur protein (Moth_1198 and Moth_1201, EC 2.1.1.245)	The CFeSP is a heterodimeric protein that contains an iron–sulfur [4Fe-4S]$^{2+/1+}$ cluster and a cobalt cobamide [32, 33]. It accepts the methyl-moiety of mTHF. The methylated CFeSP is then further used for formation of the methyl group of acetyl-CoA in the presence of the bifunctional CODH/ACS, CO and CoA. While the iron-sulfur cluster has an electron transfer function, the cobalt represents the site of methylation [46]. Reaction equation: Co(I)-CFeSP + mTHF <-> methyl-Co (III)-CFeSP + THF
Carbon-monoxide dehydrogenase (Moth_1203, EC 1.2.7.4)	The CODH, which forms a bifunctional heterotetramer with ACS [32, 33], is a homodimeric enzyme with 5 metal centers. Two [3Fe-4S] cluster, two [4Fe-4S] $^{2+/1+}$ cluster as well as one [4Fe-4S] $^{2+/1+}$ cluster bridging the two subunits [32]. It catalyzes the reduction of carbon dioxide to carbon monoxide with ferredoxin as electron carrier [32, 33, and 47]. Reaction equation: $CO + H_2O <-> CO_2 + 2H^+$
Acetyl-CoA synthase (Moth_1202, EC 1.2.99.2)	The ACS catalyzes the formation of acetyl-CoA from CO, a methyl group from methyl-CFeSP and CoA. It contains a binuclear NiNi center bridged by a cysteine residue to a [4Fe-4S] $^{2+/1+}$ cluster [32]. Reaction equation: methyl-CFeSP + CO + CoA -> acetyl-CoA
acsF (Moth_1199,) **and cooC** (Moth_1204, EC 2.1.1.245)	cooC (a Ni-binding ATPase) and acsF are both found on the *acs* gene cluster and encode proteins of unknown function. They share 44 % and 27 % homology to *Rhodospirillum rubrum* cooC [48], which facilitates nickel insertion into its CODH [32, 33, 41].
orf7 (Moth_1200)	orf 7 codes for a protein of unknown function shown to have a [2Fe-2S] ferredoxin-type iron-sulfur binding domain (source: http://www.uniprot.org/uniprot/Q2RJ75).

1.8.2 Functional expression of the target genes in *S. cerevisiae*

None of the enzymes and proteins in Table 1.4 has ever been functionally expressed in *S. cerevisiae*. To allow their functional expression, precautions considering the vitamin B_{12}-

dependency of mTR, nickel cluster assembly into CODH/ACS and the increased demand of iron-sulfur cluster assembly must be taken.

1.8.2.1 Iron-sulfur cluster assembly

S. cerevisiae is capable of iron-sulfur cluster assembly in the cytosol (ISC system) as well as in the mitochondria (CIA system) [49, 50, 51]. Enzymes and proteins that are involved in iron-sulfur cluster assembly have been identified [50] and recombinant iron-sulfur containing enzymes have already been successfully expressed in *S. cerevisiae* [52].

Nevertheless in case the existing iron-sulfur cluster assembly complex is not sufficient to meet the new demand of Fe-S assembly, this could result in the production of protein lacking the metal centers, causing them to be inactive. Jon M. Kuchenreuther et al. showed that media supplementation with additional cysteine and iron *in vivo* increased the activity of a [FeFe] hydrogenase 5 to 10 fold in an *E. coli* strain that was upregulated for the production of iron-sulfur proteins [53]. Therefore media supplementation with cysteine and iron might represent a possibility to support additional Fe-S cluster assembly. Also co-expression of the three potential maturation proteins acsF, orf7 and cooC might support iron-sulfur cluster assembly of the recombinant enzymes and proteins from *M. thermoacetica*.

1.8.2.2 Cobalamin supply for mTR

S. cerevisiae lacks the ability for vitamin B_{12} biosynthesis [54]. But medium supplementation with hydroxocobalamin enabled functional expression of the vitamin B_{12} dependent recombinant human methylmalonyl-CoA mutase in yeast [55]. According to this information, supplementation of the growth medium with vitamin B_{12} is supposed to allow functional expression of the recombinant mTR in *S. cerevisiae*.

1.8.2.3 Nickel insertion in CODH and ACS

S. cerevisiae has no system for the maturation of nickel cluster [56]. To enable nickel insertion into CODH/ACS and correct assembly of the subunits, the three potential maturation or helper proteins acsF, orf7 and cooC were co-expressed with the CODH and ACS. acsF and cooC are proteins with a high homology to cooC from *Rhodospirillum rubrum*, a membrane associated protein that participates in the maturation of the microorganisms CODH nickel cluster [41, 48]. The function of the orf7 is not yet known, but as it was also found on the *acs* gene cluster, it might also play a role in the maturation of the CODH and ACS. Based on these findings co-expression of those proteins together with additional supplementation of the

yeast cultivation medium with nickel is supposed to enable functional expression of bifunctional enzyme.

2 Aim of work

The overall aim of the thesis was to construct recombinant baker's yeast strains that enable recombinant expression of functional CODH/ACS, CFeSP and mTR from *Moorella thermoacetica*.

To achieve this, the following specific aims were defined:

1) Development of a cloning strategy suitable for multiple chromosomal gene integrations in *S. cerevisiae* based on known protocols from the literature.

2) Construction of a set of recombinant strains expressing each of the target enzymes and proteins alone or together with potential maturation proteins, as well as a strain co-expressing multiple target proteins and enzymes.

3) Analysis of resulting recombinant *S. cerevisiae* strains with respect to (i) genomic integration, (ii) transcription and (iii) functional expression.

4) Studying the effect of media supplementation with folic acid, cobalamin, cobalt and additional iron, nickel and sulfur on the functional expression of the heterologous proteins and enzymes.

3 Materials

3.1 Chemicals

TRIS, ≥99.9 %	Carl Roth GmbH + Co. KG
Acetic acid, 100 %	Carl Roth GmbH + Co. KG
EDTA, ≥99.4 %	Sigma-Aldrich
Yeast Extract	Carl Roth GmbH + Co. KG
Peptone	Carl Roth GmbH + Co. KG
α-D-(+)-Glucose Monohydrate	Carl Roth GmbH + Co. KG
Agar-agar	Carl Roth GmbH + Co. KG
NaCl, ≥99.5 %	AppliChem GmbH
Ampicillin, sodium salt, ≥99 %	Carl Roth GmbH + Co. KG
$(NH_4)_2SO_4$, ≥99 %	Sigma-Aldrich
KH_2PO_4, ≥99 %	Carl Roth GmbH + Co. KG
K_2HPO_4, ≥98 %	Sigma-Aldrich
$MgSO_4x7H_2O$	Carl Roth GmbH + Co. KG
L-Histidine, ≥98.5 %	Sigma-Aldrich
L-Leucine, ≥98 %	Sigma-Aldrich
L-Methionine, ≥98 %	Sigma-Aldrich
Uracil, ≥99 %	Sigma-Aldrich
$ZnSO_4x7H_2O$, ≥99.5 %	Sigma-Aldrich
$CoCl_2x6H_2O$, ≥98 %	Merck KGaA
$MnCl_2x2H_2O$, ≥99 %	Merck KGaA
$CuSO_4x5H_2O$, ≥98 %	Sigma-Aldrich
$CaCl_2x2H_2O$, ≥99 %	Sigma-Aldrich
$FeSO_4$ x 7 H_2O, ≥99.5 %	Merck KGaA
$Na_2MoO_4x2H_2O$, ≥99 %	Sigma-Aldrich
H_3BO_3, ≥99.5 %	Sigma-Aldrich
KI, ≥99.5 %	Carl Roth GmbH + Co. KG
Biotin, ≥98.5 %	Carl Roth GmbH + Co. KG
Calcium pantothenate, ≥98 %	Carl Roth GmbH + Co. KG
Nicotinic acid, ≥98 %	Sigma-Aldrich

Inositol, ≥99 %	Sigma-Aldrich
Thiamin HCl, ≥99 %	Sigma-Aldrich
Pyridoxine HCl, ≥98 %	Sigma-Aldrich
p-Aminobenzoic acid, ≥99 %	Sigma-Aldrich
Riboflavin, ≥98 %	Merck KGaA
Folic acid	Serva
$MgCl_2$ x $6H_2O$, ≥99 %	Merck KGaA
KCl, ≥99.5 %	Merck KGaA
Glycerol, ≥99 %	Sigma-Aldrich
Ethanol, 99.9 %	Merck KGaA
NaOH, ≥99 %	Carl Roth GmbH + Co. KG
dATP [100 mM]	Fermentas International Inc.
dTTP [100 mM]	Fermentas International Inc.
dGTP [100 mM]	Fermentas International Inc.
dCTP [100 mM]	Fermentas International Inc.
peqGOLD Universal Agarose	Peqlab
Gel Red Nucleic Acid Stain	Biotium
HDGreen Plus DNA stain	INTAS
Lithium acetate dehydrate, ≥63 %	Sigma-Aldrich
PEG_{3640}	Sigma-Aldrich
Single stranded salmon sperm [10 mg/ml]	Sigma-Aldrich
D-(+)-Galactose, ≥99 %	Sigma-Aldrich
Vitamin B_{12}, ≥98 %	Sigma-Aldrich
L-cysteine, ≥99 %	Carl Roth GmbH + Co. KG
$NiCl_2$ x $6H_2O$, 98 %	Sigma-Aldrich
Bovine serum albumin (BSA), ≥95 %	Sigma-Aldrich
Roti®-Quant	Bio-Rad Laboratories
Sodium acetate anhydrous, ≥99 %	Sigma-Aldrich
NuPAGE® Novex® 4-12 % Bis-Tris Mini Gel	Life Technologies
NuPAGE® Reducing Agent (10x)	Life Technologies
NuPAGE® LDS Sample Buffer	Life Technologies
$Na_2S_2O_3$, 99 %	Sigma-Aldrich
Sodiumacetate, ≥98.5 %	Carl Roth GmbH + Co. KG
Tetrahydrofolic acid, 65 %	Sigma-Aldrich

Methylcobalamin, ≥97 %	Sigma-Aldrich
Dithiothreitol (DTT), ≥97 %	Sigma-Aldrich
Methylviologen dichloride hydrate, 98 %	Sigma-Aldrich
Carbon monoxide, ≥99 %	Sigma-Aldrich
Coenzyme A Trilithium Salt Dihydrate, 96 %	Fisher Scientific
Ethylene glycol, 99.8 %	Sigma-Aldrich
Iodomethane, ≥99 %	Sigma-Aldrich
Ultra pure water	TKA GenPure ultra pure water system

3.2 Enzymes / Buffer

FastDigest Spel [10 U/µl]	Thermo Scientific
FastDigest XhoI [10 U/µl]	Thermo Scientific
T4 DNA Ligase [5 U/µl]	Fermentas International Inc.
Clal FastDigest	Thermo Scientific
Hindlll FastDigest	Thermo Scientific
DreamTaq polymerase [5U/µl]	Thermo Scientific
10x DreamTaq buffer	Thermo Scientific
Phusion Polymerase [2 U/µl]	Thermo Scientific
5x Phusion® HF Buffer	Thermo Scientific
DMSO	Thermo Scientific
GoTaq Polymerase [5 U/µl]	Fermentas International Inc.
6x DNA Loading Dye + SDS	Fermentas International Inc.
GeneRuler 1 kb DNA Ladder	Thermo Scientific
GeneRuler 1 kb PLUS DNA Ladder	Thermo Scientific
DNase I, RNase-free [1 U/µl]	Thermo Scientific
Lyticase/Zymolase [≥2.000 U/mg protein]	Sigma-Aldrich
Protease Inhibitor Cocktail	Sigma-Aldrich
Zymolyase [5 U/µl]	Zymo Research
10x FastDigest® Green buffer	Thermo Scientific
PageRuler Prestained Protein Ladder	Thermo Scientific

3.3 Kits

GeneJET PCR Purification Kit	Thermo Scientific
GeneJET Gel Extraction Kit	Thermo Scientific
GeneJET Plasmid Miniprep Kit	Thermo Scientific
GeneJET RNA Purification Kit	Thermo Scientific
RevertAid H Minus First Strand cDNA Synthesis Kit	Thermo Scientific
QuantiFast SYBR Green PCR kit	Qiagen

3.4 Instruments

Thermal Cycler I Cycler	Bio-Rad Laboratories
Thermal Cycler My CyclerTM	Bio-Rad Laboratories
Thermomixer Comfort	Eppendorf AG
Centrifuge “Eppifuge” 5415R	Eppendorf AG
Centrifuge 5804R	Eppendorf AG
Beckman JA-10 Centrifuge	Beckman Coulter
SorvallR RC-5B Refrigerated Superspeed Centrifuge	Thermo Fisher Scientific
Micro PulserTM	Bio-Rad Laboratories
Electroporation Cuvettes LE; 2 mm Electrode gap	Peqlab Biotechnologie GmbH
Gel-Doc 2000	Bio-Rad Labaratories
pH Meter 420A	Orion Thermo Electron Corporation
Sartolon Polyamide Filter 0.2 μm	Satorius AG
MF-Millipore™ Membrane Filters	Millipore
Spectrophotometer DU800	Beckman Coulter Inc.
RNase-free Microfuge Tubes (1.5 ml)	Lifetechnologies
WPA CO 8000 Cell Density Meter	Biowave
Glove Box	Plas Labs
DS 11+ Spectrophotometer	DeNovix
0.5 mm Glass Beads	BioSpec
Semi-Micro Spectrophotometer Cells, Screw Top	Starnacells
TG-320 digital camera	Olympus

HPLC/MS system	Thermo Fisher Scientific
HPLC Dionex Ultimate3000	
Autosampler WPS-3000	
Solvent rack SRD-3600	
Pump LGP-3600 (LPG-3000)	
Flow manager FLM-3300	
Pre-column Atlantis T3 C18, 3 µM, 100 A, 10 x 2.1 mm	Waters Corporation
Column Atlantis T3 C18, 3 µm, 100 A, 150 x 2.1 mm	Waters Corporation
HPLC vials, 200 µL , TopSert TPX-Short Thread Vial, 32x11.6	VWR
Heated electrospray ionization source (HESI)	Thermo Fisher Scientific
Mass spectrometer Exactive™	Thermo Fisher Scientific

3.5 Software

IDT Oligo Analyzer Software
RegRNA 2.0
Xcalibur software, version 2.2 SP1
Basic Local Alignment Search Tool (BLAST) - NCBI

3.6 Buffer

3.6.1 50x TAE buffer

242 g TRIS, 57.1 ml acetic acid and 18.6 g EDTA were dissolved in 1 liter ddH_2O and adjusted to a pH of 8.0.

3.6.2 50 mM potassium phosphate buffer

50 mM potassium phosphate buffers with a pH of 6.8, 7.0 or 7.4 were prepared. Therefore 1M monopotassium phosphate (KH_2PO_4) and 1M dipotassium phosphate (K_2HPO_4) with the respective volumes listed in Table 3.1 were added to in 950 ml ddH_2O and mixed.

Table 3.1: Production of potassium phosphate buffer. The final volume of the buffer was 1000 ml.

Potassium phosphate buffer [50mM]	1M KH_2PO_4	1M K_2HPO_4
pH 6.8	26.5 ml	23.5 ml
pH 7.0	21.1 ml	28.9 ml
pH 7.4	11.2 ml	38.8 ml

3.6.3 1x SDS running buffer

The 1x SDS Running Buffer contained 50 ml 20x NuPAGE® MOPS SDS Running Buffer in 950 ml ddH_2O.

3.6.4 Iodomethane

Iodomethane (ICH_3) was purchased from Sigma Aldrich. It arrived at a concentration of 2.25 g/ml [16 mol/L]. A stock solution was prepared with ddH_2O with a concentration of 14.19 mg/ml [1 mol/L]. Fresh stock solutions were prepared before each use.

3.7 Silver staining solutions

Fixation solution

The fixation solution contained 10 % (v/v) acetic acid and 40 % (v/v) ethanol in water.

Sensitizing solution

The sensitizing solution contained 0.2 % sodiumthiosulfate (w/v), 0.5 M sodiumacetate (w/v) and 30 % (v/v) ethanol dissolved in water.

Silver solution

The silver solution contained 0.2% (w/v) silver nitrate in H_2O.

Developing solution

The developing solution contained 3 % (w/v) sodium carbonate and 0.01 % (v/v) formaldehyde dissolved in water.

Stopping solution

The stopping solution contained 1.5 % EDTA (w/v) dissolved in water.

3.8 Plasmids

The shuttle vectors pXP314, pXP318, pXP320, pXP322, pBF3038 and pBF3060 were purchased from Addgene and arrived in the form of bacterial stab cultures (DH5alpha), which were stored at 4 °C for 2 weeks. The vector map and general information about the shuttle vectors pXP314, pXP318, pXP320 and pXP322, are shown in Table 3.2. Vector pBF3038 and pBF3060 both harbor a CreA recombinase. The respective vector maps and properties are shown in Table 3.3.

Table 3.2: Yeast shuttle vector. Vector map and information about the yeast shuttle vectors used.

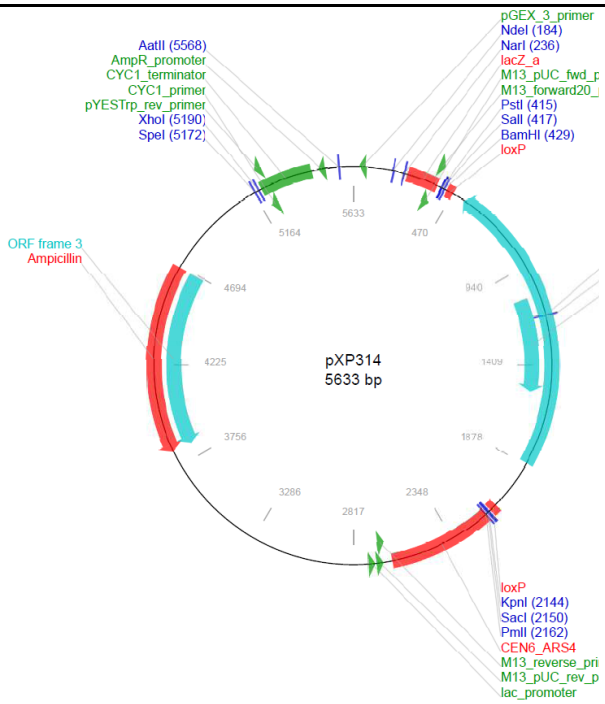
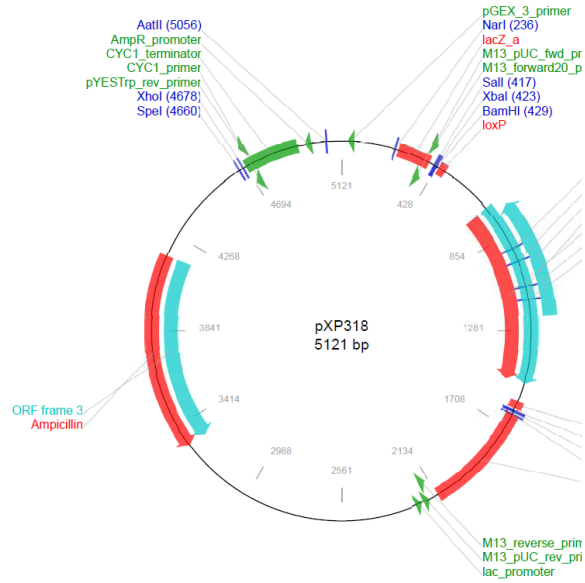
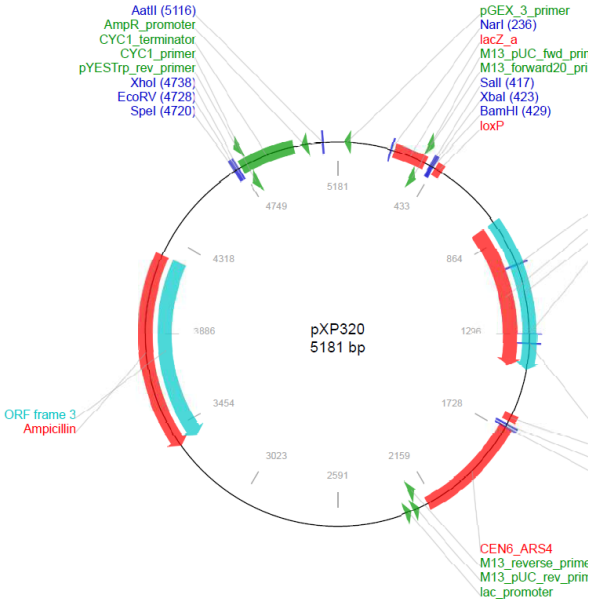
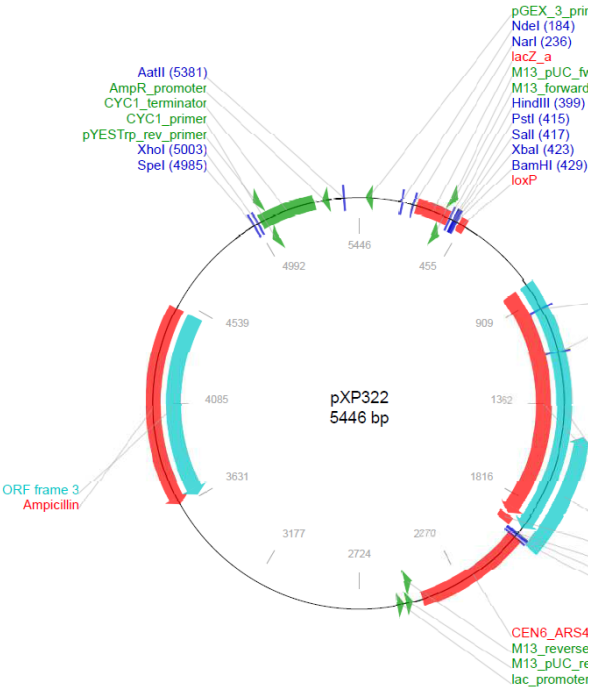
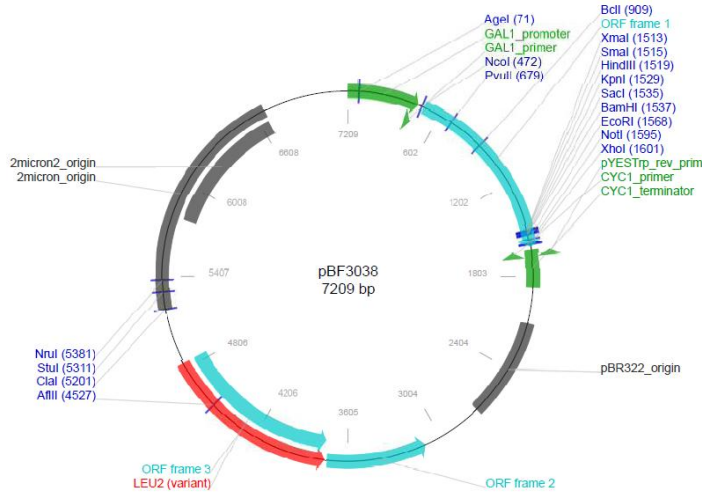
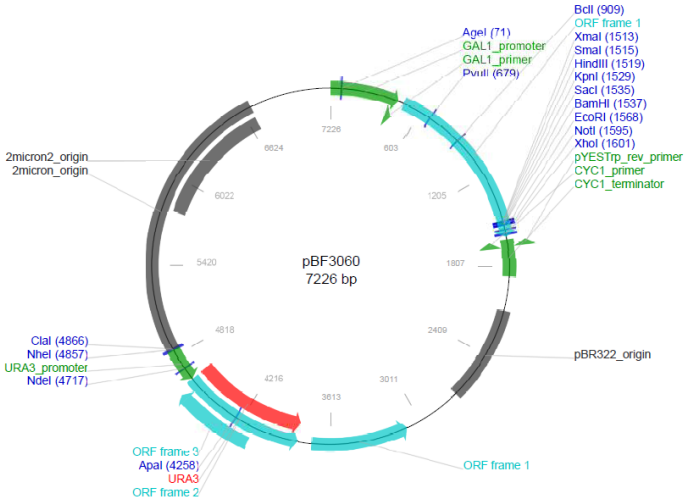
Vector name	Vector information [a]	Selection marker
pXP314		Met15
pXP318		URA3

Table 3.2: (Continued)

Vector name	Vector information [a]	Selection marker
pXP320		HIS3
pXP322		LEU2

[a] vector backbone: pUC18, bacterial resistance: ampicillin, growth temperature: 37°C, growth strain: DH5alpha, copy number: high copy, two *loxP* sites to facilitate removal of the auxotrophic markers via CreA recombinase, CYC1 teminator, TEF1 promoter, 5′ SpeI and 3′ XhoI cloning site downstream of the TEF1 promoter. Source: https://www.addgene.org

Table 3.3: CreA expression vectors. Vector maps and information about the vectors used for the marker rescue.

Vector name	Vector information [a]	Selection marker
pBF3038		LEU2
pBF3060		URA3

[a] vector backbone: pYES2.0, bacterial resistance: ampicillin, growth temperature: 37°C, growth strain: DH5alpha, copy number: high copy, insert: CreA recombinase, promoter: GAL1, terminator: CYC1, origin: 2micron2 and pBR322. Source: https://www.addgene.org

The 8 target genes were purchased from Biomatik and were codon optimized for expression in *S. cerevisiae* by the company with 15 % cut off for codon efficiency. The vector maps and their properties are shown in Table 3.4. Secondary structures were analyzed by Biomatik using a build in M-fold module and internal ribosomal binding sites were removed. Additional SpeI (5’ ACTAGT 3’) and XhoI (5’ CTCGAG 3’) restriction sites were added at the 5’and 3’end of the genes. SpeI and XhoI restriction sites in the genes were removed. All genes were provided in a pBMH vector except the mTR gene which was provided in a pJET1.2 vector. All vectors carried the bacterial resistance ampicillin.

The purchased plasmids containing the target genes arrived lyophilized (4µg) and were dissolved in 40 µl filter sterilized ddH_2O to a concentration of 100 ng/µl. These stock solutions were stored at -20 °C.

Table 3.4: Vectors containing *M. thermoacetica* genes.

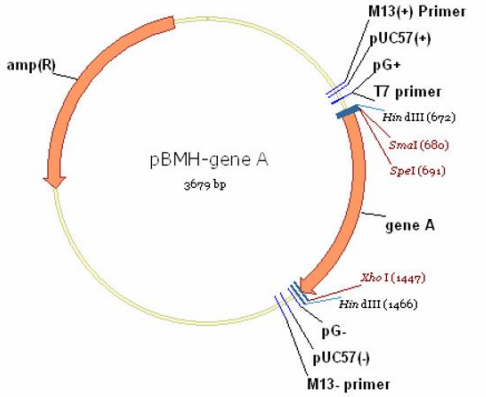
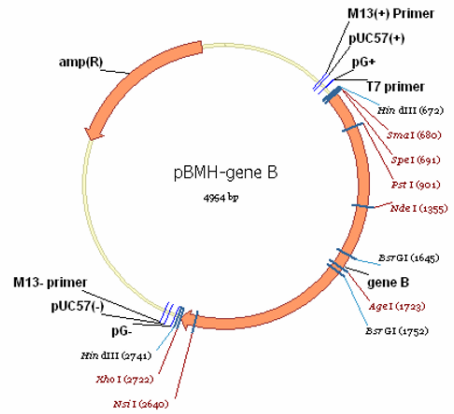
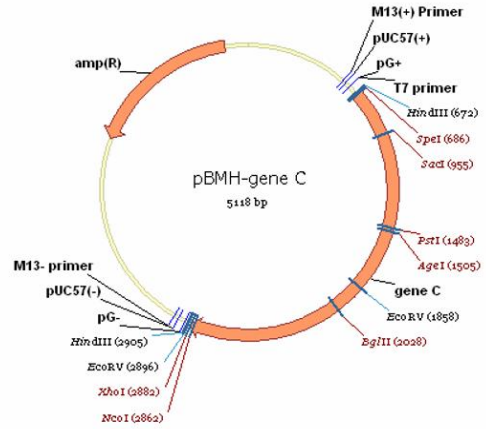
Vector name	Vector information [a]	Gene name	Gene size [bp]	Biomatik order name
pBMH-Gene A		Moth_1204 (cooC)	762	SG1130382-1
pBMH-Gene B		Moth_1203 (CODH)	2037	SG1130382-2
pBMH-Gene C		Moth_1202 (ACS)	2202	SG1130382-3

Table 3.4: (Continued)

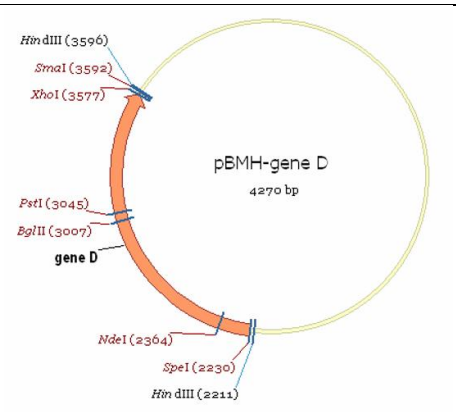
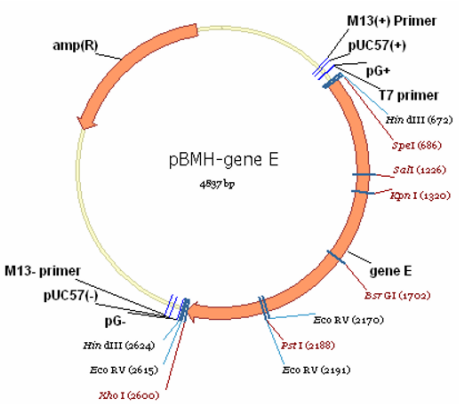
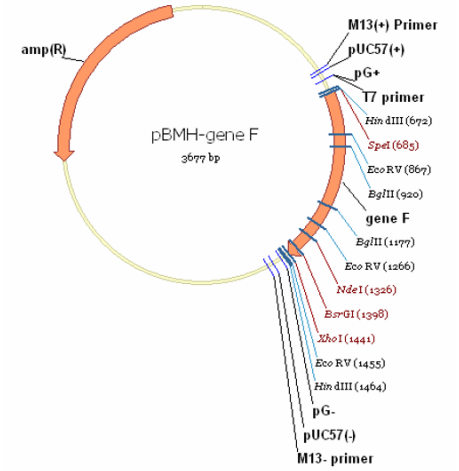
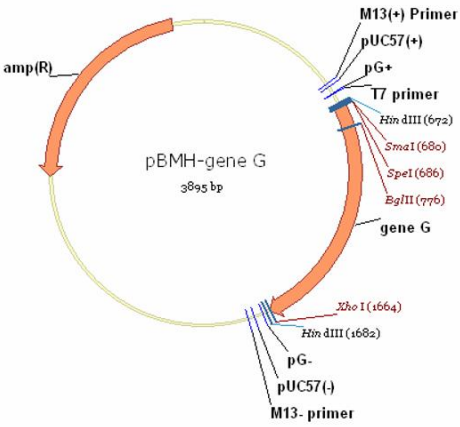
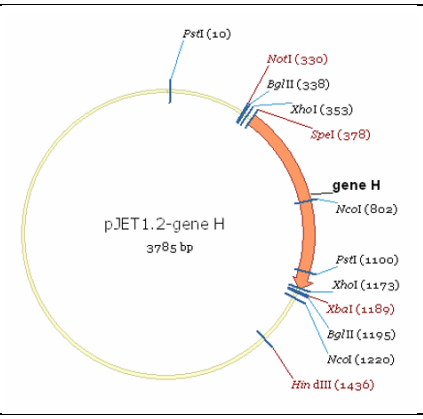
Vector name	Vector information [a]	Gene name	Gene size [bp]	Biomatik order name
pBMH-Gene D		Moth_1201 (CFeSP large)	1353	SG1130382-4
pBMH-Gene E		Moth_1200 (orf7)	1920	SG1130382-5
pBMH-Gene F		Moth_1199 (acsF)	762	SG1130382-6
pBMH-Gene G		Moth_1198 (CFeSP small)	984	SG1130382-7

Table 3.4: (Continued)

Vector name	Vector information [a]	Gene name	Gene size [bp]	Biomatik order name
pJET1.2 -Gene H		Moth_1197 (mTR)	801	SG1130382-8

[a] Source: www.biomatik.com

3.9 Primer

Primers were designed using the IDT Oligo Analyzer Software. They were purchased at Sigma Aldrich. Information about sequence and melting temperature of the oligonucleotides used are listed in Table 3.5 to 3.8.

Table 3.5: Primer pairs used to control ligation. REV and FWD indicating forward and reverse primer respectively.

Primer Name	Primer sequence	Tm [°C]
TEF1 FWD	5'-CAC CCA ATC CCC CAC AAG TGA-3'	71.2
Moth_1204 REV	5'-AAATGATTGAGTAGAAGTAATTCCAGC-3'	62.5
Moth_1203 REV	5'-AGT TTC GTA TCT CTC TGC GAC TTC-3'	63.8
Moth_1202 REV	5'-GGT TGT ACC TAT TGT TTC GTC TGC-3'	64.4
Moth_1201 REV	5'CGGCACCTGGAATAATGATTCTATG3'	67.8
Moth_1200 REV	5'-GCC AAT TCA GCA CCC TTC AAT GA-3'	70.9
Moth_1199 REV	5'-TTA CTC CAG TCA ATG GAA TAC CAG TTC-3'	65.8
Moth_1198 REV	5'-CCT TAA CTC TAG CAA CTG CTT CAG-3'	62.3
Moth_1197 REV	5'-TAG CAG CAT CCA ATC CAC ATG-3'	65.9

Table 3.6: Primer pairs used to construct linear expression cassettes. REV and FWD indicating forward and reverse primer respectively. Loci homologous sequences are colored in yellow. TEF1 promoter homologous sequence are colored in green and the *loxP* homologous sequence are colored in turquoise.

Primer Name	Primer sequence	Tm [°C]
TRP1-D-FWD	5'- TGC ACC CGC CCG TCT GGA CGC GCC GCT CAC CCG CAC GGC AGA GAC ACC GCG AAT CCT TAC ATC AC-3'	67.3
TRP1-D-REV	5'- TAT TTT ACA GAT TTT ATG TTT AGA TCT TTT ATG CTT GCT TTT CAA AAG GCA TTC GAG CTC GGT AC -3'	80.8
YLRCTy1-1-C-FWD	5'- ACA GAG AGC ACG GTT GCG AAC GGA GGG TCT CAC AAT GTC TCA AAG ACC GCG AAT CCT TAC ATC AC-3'	87.6
YLRCTy1-1-C-REV	5'- ACA ATC ATA TCT TGT ATG CGG CCC GCA AAC CAA GAG ATT TAT CCT TTG TGA TTC GAG CTC GGT AC -3'	91.2
YBLMTy1-1-B-FWD	5'- TAT TAT CTG TAT ATC TAA TAT TAT AGT CTC TAA CAA CAG TGG AAT ACC GCG AAT CCT TAC ATC AC -3'	76.3
YBLMTy1-1-B-REV	5'- ACA ATC ATA TCT TGT ATG CGG CCC GCA AAC CAA GAG ATT TAT CCT TTG TGA TTC GAG CTC GGT AC -3'	87.9
YDRWTy1-5-G-FWD	5'- CAC AGA GTT GTA TTT GCG CTT CTG AGC GAT GCT TCC GAG ATT GTT ACC GCG AAT CCT TAC ATC AC -3'	89.5
YDRWTy1-5-G-REV	5'- TAT TGA AGA GGG ATG CGT TTG GTA CAA TAA AAA ACA TAG GTT CCC AAA CCA TTC GAG CTC GGT AC -3'	85.8
YMLWTy1-2-H-FWD	5'- GGA ACT CTA AAA TAT CAT TTG TTT AGT AGT ATT CGT GTT ACT AGT ACC GCG AAT CCT TAC ATC AC -3'	78.3
YMLWTy1-2-H-REV	5'- TAA CGA AAC AGA ATG AGC ATC CTT ACA CAA TTT TAA CAT TCA TCT ATT CGA GCT CGG TAC -3'	81.8
YPRCTy1-2-A-FWD	5'-AAT ATG ATA GCC TTT ACC AAC AAT GGA ATC CCA ACA ATT ATC GAA TTA ACC GCG AAT CCT TAC AT-3'	83.4
YPRCTy1-2-A-REV	5'-CCG GTA TTT ATT TCT TTG CAA CCA AAA TAT GGA TAT CGA GAT GTA TTT GA ATTCGAGCTC GGTAC-3'	83.3
YMLWTy1-1-E-FWD	5'-CGG GAA CGG GCA ACA AAG GTA ATG TTT ATA TGA CCG CGA ATC CTT ACA TCA C-3'	85.4
YMLWTy1-1-E-REV	5'-CTT CAT AGA GTA ATA TTT TGC ATA TTG ATA ATA AAG AAC ATA GCA TAT ATT CGA GCT CGG TAC-3'	76.2
YILWTy3-1-F-FWD	5'- ACA GGG TTT GTA GCA CTG GTA TAC GTT TTT CCA TAT CAT GGA ACA ACC GCG AAT CCT TAC ATC AC -3'	86.4
YILWTy3-1-F-REV	5'- GCA CGG TGC CTT AAC CAA CTG GGC CAA GAG ACC AGA TAT TTC ATA TGT TCA TTC GAG CTC GGT AC -3'	89.1

Table 3.7. Primer pairs used to control genomic integration. REV and FWD indicating forward and reverse primer respectively.

Primer Name	Gene	Primer sequence	Tm [°C]
giYPRCTy1-2- FWD	Moth_1204	5'-ATG ACT CCC TGA TAT GCG AAT-3'	62.2
Moth_1204 REV		5'-AAATGATTGAGTAGAAGTAATTCCAGC-3'	62.5
giYBLMTy1-FWD	Moth_1203	5'-TGT GCG TGT TCA TAA GGG AAA-3'	65.2
Moth_1203 REV		5'-AGT TTC GTA TCT CTC TGC GAC TTC-3'	63.8
giYLRCTy1-FWD	Moth_1202	5'-AGC ATA TCA GTC GAA TGA AGT TCC-3'	64.2
Moth_1202 REV		5'-GGT TGT ACC TAT TGT TTC GTC TGC-3'	64.4
giTRP1- FWD	Moth_1201	5'-GGA CAG TTA GAG GCG GTG GA-3'	66.4
Moth_1201 REV		5'-CGGCACCTGGAATAATGATTCTATG-3'	67.8
giYMLWTy1-1- FWD	Moth_1200	5'-GCG ATC TTT ATT GGT ATT TGT TGG-3'	64.1
Moth_1200 REV		5'-GCC AAT TCA GCA CCC TTC AAT GA-3'	70.9
giYILWTy3-1- FWD	Moth_1199	5'-GTG GTC ATC GTC GCC TCT ACT-3'	65.5
Moth_1199 REV		5'-TTA CTC CAG TCA ATG GAA TAC CAG TTC-3'	65.8
giYDRWTy1-FWD	Moth_1198	5'-ATC GGT AGC CTC ATC TGG GAT-3'	65.8
Moth_1198 REV		5'-CCT TAA CTC TAG CAA CTG CTT CAG-3'	62.3
giYMLWTy1-2FWD	Moth_1197	5'-GCC CTT GTG AAC TTG TGA ATC TTA-3'	65.1
Moth_1197 REV		5'-TAG CAG CAT CCA ATC CAC ATG-3'	65.9

Table 3.8: Primer pairs used for qPCR. REV and FWD indicating forward and reverse primer respectively.

Primer Name	Gene	Primer Sequence	Tm [°C]
AqPCRMoth_1204 FWD	Moth_1204	5'- TCA TAG GAA ACA AGT TGA GAC ATC -3'	61
Moth_1204 REV		5'-AAATGATTGAGTAGAAGTAATTCCAGC-3'	61
BqPCRMoth_1203 FWD	Moth_1203	5'-GCA TCA GAC GTA TAT GGT GGT TAT -3'	62.6
Moth_1203 REV		5'-AGT TTC GTA TCT CTC TGC GAC TTC-3'	56.3
CqPCRMoth_1202 FWD	Moth_1202	5'-AGC AGA TGG TGG TAT AGC AAG -3'	60.7
Moth_1202 REV		5'-GGT TGT ACC TAT TGT TTC GTC TGC-3'	56.0
DqPCRMoth_1201 FWD	Moth_1201	5'-GGT TTA TCT GTT TTG ACC GCT T -3'	63
Moth_1201 REV		5'CGGCACCTGGAATAATGATTCTATG3'	55.7
EqPCRMoth_1200 FWD	Moth_1200	5'- TCC CAA ATG CTG TTG AAA TAG GAT -3'	66.2
Moth_1200 REV		5'-GCC AAT TCA GCA CCC TTC AAT GA-3'	58.2
FqPCRMoth_1199 FWD	Moth_1199	5'-TGA TGC TTC TGC TAG AGG TAT TAG -3'	60.5
Moth_1199 REV		5'-TTA CTC CAG TCA ATG GAA TAC CAG TTC-3'	56.4
GqPCRMoth_1198 FWD	Moth_1198	5'-CTG GAT GGG GTA AGG AAA CTG -3'	64.4
Moth_1198 REV		5'-CCT TAA CTC TAG CAA CTG CTT CAG-3'	55.4
HqPCRMoth_1197 FWD	Moth_1197	5'- TGC TCC AGA GGT TTT GAA GAC -3'	63.3
Moth_1197 REV		5'-TAG CAG CAT CCA ATC CAC ATG-3'	55.2

3.10 Growth media

3.10.1 Yeast peptone dextrose medium (YPD)

10 g yeast extract and 20 g peptone were dissolved in 800 ml distilled water and autoclaved. 20 g glucose were dissolved in 200 ml distilled water and autoclaved separately. After sterilization both solutions were pooled and the medium was stored at 4 °C before further use.

3.10.2 Luria-Bertani medium (LB)

5 g yeast extract, 10 g peptone and 5 g NaCl were dissolved in 1 liter distilled water. The medium was autoclaved and stored at 4 °C before further use. When needed for selection, 115 µg/ml ampicillin were added to the LB medium after the medium had cooled down to approximately 55 °C after sterilization. The medium was stored at 4 °C before further use.

3.10.3 Defined mineral medium

10 g $(NH_4)_2SO_4$, 28.8 g KH_2PO_4, 1 g $MgSO_4$ x $7H_2O$ and 1 ml trace element were dissolved in 800 ml distilled water and the pH was adjusted to 6.5. 20 g/l glucose were dissolved in 200

ml distilled water. Both solutions were autoclaved and pooled after sterilization. After the medium had cooled to approximately 55 °C, 1 ml vitamin solution were sterile filtered (cut-off 0.2 µm) and added to the sterile medium.

After sterilization L-methionine, L-histidine or uracil was added at a final concentration of 20 mg/l and L-leucin to a final concentration of 30 mg/l. The supplements were dissolved in distilled water, sterile filtered (cut-off 0.2 µm) and added to the sterile medium.

The trace element solution contained:
0.45 g/l $ZnSO_4x7H_2O$
0.03 g/l $CoCl_2x6H_2O$
0.082 g/l $MnCl_2x2H_2O$
0.03 g/l $CuSO_4x5H_2O$
0.45 g/l $CaCl_2x2H_2O$
0.3 g/l $FeSO_4x7H_2O$
0.04 g/l $Na_2MoO_4x2H_2O$
0.1 g/l H_3BO_3
0.01 g/l KI
1.5 g/l EDTA

The vitamin solution contained:
0.005 g/l Biotin
0.1 g/l Calcium pantothenate
0.1 g/l Nicotinic acid
2.5 g/l Inositol
0.1 g/l Thiamin HCl
0.1 g/l Pyridoxine HCl
0.02 g/l p-Aminobenzoic acid
0.02 g/l Riboflavin
1 ml/l Folic acid [20mg/100ml]

The solutions were sterilized by filtration (cut-off 0.2 µm), portioned and stored at 4 °C before application to the appropriate medium.

3.10.4 Agar-plates

For the preparation of YPD, LB or mineral medium agar-plates, 16 g/l agar-agar were added to the respective medium before sterilization. After autoclaving the medium was cooled to approximately 55 °C. For the preparation of LB-amp plates 115µg/ml ampicillin were added after cooling of the medium. For the preparation of mineral medium selection plates sterile filtered L-methionine, L-histidine or uracil (at a final concentration of 20 mg/l) and L-leucine (to a final concentration of 30 mg/l) were added for the preparation of mineral medium selection plates. The cooled medium was then poured into petri-dishes. The plates were hardened at room temperature and then stored at 4 °C before further use.

3.10.5 SOC medium

For the preparation of SOC medium 10 g peptone, 2.5 g yeast extract, 1.02 g $MgCl_2$ x $6H_2O$, 0.29 g NaCl and 0.09 g KCl were dissolved in 800 ml distilled water. The pH was adjusted to 7.0. 1.8 g Glucose was dissolved in 200 ml distilled water. The solutions were autoclaved and pooled after cooling to room temperature and stored at 4 °C before further use.

3.10.6 YPG medium

For the preparation of YPG medium 10 g yeast extract and 20 g peptone were dissolved in 800 ml distilled water and autoclaved. 20 g galactose were dissolved in 200 ml distilled water and autoclaved separately. After sterilization both solutions were pooled and the medium was stored at 4 °C for not more than a week before further use.

3.10.7 1% agarose gel

For the preparation of a 1% agarose gel 1 g peqGOLD Universal Agarose was dissolved in 100 ml TAE buffer in a microwave. After heating, the mixture was cooled down to around 55 °C on the benchtop before 5 µl nucleic acid stain (Gel Red Nucleic Acid Stain from Biotium or HDGreen Plus DNA stain from INTAS) was added. Then the gel was swirled and poured into a casting tray where it was hardened.

3.11 Cultivations

3.11.1 Strains

Saccharomyces cerevisiae

BY4741; MATa; his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0	Euroscarf

The yeast strain BY4741 was derived from *Saccharomyces cerevisiae* S288C [57]. 4 selectable markers were deleted in this strain in order to minimize the homology between the sequence of the markers in the genome and the marker sequences used in cloning vectors.

Escherichia coli

E.coli Top 10	Invitrogen

4 Methods

4.1 Strain cultivation

4.1.1 *S. cerevisiae* BY4741

The wild type strain was grown aerobically and anaerobically at 30 °C in YPD medium or mineral medium containing L-methionine, L-histidine, L-leucine and uracil. Transformed *S. cerevisiae* BY4741 strains were grown aerobically and anaerobically at 30 °C in mineral medium lacking either one or combinations of L-methionine, L-histidine, L-leucine and uracil allowing selection of transformed strains. Aerobic cultivations were carried out in 250 ml shake flasks (working volume: 100 ml) at 120 rpm. Anaerobic cultivations were carried out in 100 ml cultivation bottles sealed with open top screw caps (working volume: 60 – 80 ml) at 180 rpm, or on respective agar-plates.

4.1.2 *E.coli*

E.coli Top 10 cells used for the preparation of electro competent cells were grown in LB medium at 37 °C in 250 ml shake flasks (working volume: 100 ml) at 150 rpm.
E.coli Top 10 and DH5alpha cells containing plasmids with ampicillin resistance were grown in LB medium containing ampicillin at 37 °C in 250 ml shake flasks (working volume: 100 ml) at 150 rpm or on agar-plates containing the respective antibiotic.

4.1.3 Preparation of overnight cultures (ONCs)

S. cerevisiae BY4741
A single colony of *S. cerevisiae* BY4741 or recombinant form thereof, obtained from agar-plate cultivations (incubation time around 48 hours) was used to inoculate ONCs which were carried out in 50 ml YPD medium in 150 ml shake flasks and grown at 30 °C and 120 rpm. For anaerobic cultivation a single *S. cerevisiae* BY4741 colony was transferred to a 100 ml rubber stoppered glass serum bottle containing 50 ml YPD medium inside the glove box. Then the inoculated medium was flushed with sterile nitrogen (using a cut-off 0.1 µm membrane filter) for 30 minutes. Cultivations were performed at 180 rpm at 30 °C.

E.coli

A single *E.coli* Top 10 or DH5alpha colony was used to inoculate 50 ml LB medium in 150 ml shake flasks and grown at 37 °C and 150 rpm. Transformed cells carrying plasmids with ampicillin resistance were grown in LB medium containing ampicillin.

4.2 Storage of microorganisms and plasmids

4.2.1 Glycerol stocks

First ONCs were prepared from a single *E.coli* Top 10, DH5alpha, *S. cerevisiae* BY4741 or transformed strain colonies. After reaching the stationary phase, 500 µl of those ONCs were added to 500µl of sterile 50 % (v/v) aqueous glycerol in a sterile 1.5 ml Eppendorf tube and mixed thoroughly. The glycerol stock was then immediately frozen at – 80 °C.

E.coli Top 10, competent and transformed *E.coli* Top 10 cells and *E.coli* DH5alpha cells as well as the *S. cerevisiae* BY4741 and all transformed yeast strains were stored as glycerol stocks at -80 °C.

4.3 Molecular biology procedures

All materials including solutions, pipette tips and Eppendorf tubes that were used for the molecular biological methods were sterilized before use.

4.3.1 *E. coli* Top 10 transformation

10 ng plasmid were added to an Eppendorf tube containing 50 µl electro-competent *E. coli* Top 10 cells, which were thawn on ice, and cooled on ice for 20 min. Before electroporation the cell/plasmid mixture was transferred into electroporation cuvettes that were pre-cooled on ice. Electroporation was conducted using the Micro PulserTM program EC2. After electroporation the cells were immediately transferred to 1 ml of at 37 °C pre-heated SOC medium and cultivated at 37 °C and 300 rpm for 1 hour using an Eppendorf thermomixer. 10 µl, 50 µl and the rest which was obtained after brief centrifugation and supernatant discharging, were plated onto LB agar-plates containing ampicillin. Plates were incubated at

37 °C overnight for around 14 hours. Single colonies were counted and archived for maximum one month on a master LB plate containing ampicillin. As negative control 2 µl ddH_2O were transformed.

4.3.2 Preparation of *E. coli* Top 10 electro competent cells

E. coli Top 10 cells from a glycerol stock were streaked onto LB plates and grown for about 14 hours at 37 °C. One of the obtained single colony was selected from the fresh LB plate and used to inoculate 10 ml LB medium and cells were cultivated at 37 °C and 150 rpm in a shaker for about 14 hours for the preparation of a starter culture. On the next day four 1000 ml shake flasks with each 250 ml LB medium were inoculated with starter culture to a final OD_{600} of 0.05. The cells were grown to an OD_{600} of 0.4 and then the shake flasks were put on ice. The cell cultures were poured into ice cold sterile centrifuge bottles and centrifuged at 5000 rpm in a precooled Beckman JA-10 centrifuge for 20 minutes at 4 °C. The supernatant was discharged and the cell pellets were resuspended in 100 ml ice cold ddH_2O with a pipette. The cells were centrifuged at 4000 rpm for 10 minutes at 4 °C. The supernatant was discharged and the cell pellets were again resuspended in 100 ml ice cold ddH_2O. The suspended cell materials were pooled and the cells collected by centrifugation at 4000 rpm for 20 minutes at 4 °C. The supernatant was discharged and the cell pellets resuspended in 10 ml ice cold solution containing ddH_2O and 10 % (v/v) glycerol. The cells were centrifuged at 2100 rpm for 20 minutes at 4 °C, the supernatant removed and the pellet resuspended in ice cold 10 % glycerol to a final OD_{600} of 200. 50 µl of the cells were then transferred into sterile 1.5 ml Eppendorf tubes and shock frozen in liquid nitrogen. Electrocompetent cells were stored at -80 °C.

4.3.3 Determination of cell concentration

The cell concentration of *S. cerevisiae* wild type and transformed strains as well as of *E. coli* Top 10 and DH5alpha cultures was determined by measurement of the optical density (OD) at 600 nm (OD_{600}) on a Beckman Coulter DU 800 UV/Vis Spectrophotometer. Samples were diluted with the respective growth medium, if necessary, to measure in the working range of 0.1 to 1.0 of the Spectrophotometer.

4.3.4 Determination of DNA/RNA content

The DNA concentration in samples was measured with a DS 11+ Spectrophotometer. RNA concentration was measured using the DS 11 + Spectrophotometer from DeNovix.

4.3.5 Determination of protein content

The protein content was determined via the Bradford assay using Roti®-Quant 1:5-fold diluted with ddH_2O as dye reagent.
At first a calibration curve was established with bovine serum albumin in a concentration range of 0.1 to 1.0 mg/ml. Therefore five dilutions with concentrations of 0.1, 0.25, 0.5, 0.75 and 1 mg/ml of the BSA standard were prepared.

For the assay 20 µl of the protein solution (BSA standard solution or cell-free extract) were added to 1 ml of the diluted reagent and mixed. The mixture was incubated at room temperature for 15 minutes and then the absorbance was measured at 595 nm. The measurements were always carried out in duplicates and for the blank 20 µl water instead of protein solution were used.

4.3.6 Plasmid propagation

Plasmids containing *M. thermoacetica* genes were propagated in *E. coli* Top 10 cells while yeast shuttle vectors (pXP314, pXP318, pXP320, pXP322, pBF3038 and pBF3060) were propagated in *E. coli* DH5alpha cells. Detailed information about the plasmids used can be found in Tables 3.2, 3.3 and 3.4 in the Material part Section 3.6.

Electro competent *E.coli* Top 10 cells were transformed with 1 ng plasmid. Transformed colonies were grown on LB medium containing ampicillin at 37 °C. For proliferation of the shuttle vectors, *E. coli* DH5alpha cells containing the respective shuttle vector were spread on LB-amp plates and incubated at 37 °C. Obtained single colonies from the transformed *E.coli* Top 10 and the different DH5alpha cells were used for the preparation of ONCs for the production of glycerol stocks and for further plasmid isolation via Miniprep.

4.3.7 Isolation of amplified plasmids

Plasmid isolation was carried out by using the GeneJET Plasmid Miniprep Kit and following the instructions in the manual.

4.3.8 Plasmid double digestion with SpeI and XhoI

To cut out the target genes from the plasmids in which they were shipped, and to prepare the shuttle vectors for gene integration a double digest with the restriction enzymes Spel and XhoI was conducted from vector pBMH-Gene A, pBMH-Gene B, pBMH-Gene C, pBMH-Gene D, pBMH-Gene E, pBMH-Gene F, pBMH-Gene G, pJET1.2-Gene H, pXP314, pXP318, pXP320 and pXP322.
The reaction mixture contained 2 µl 10x FastDigest® Green buffer, 1 µl XhoI [10 Units] and 1 µl SpeI [10 Units] Fast-digest, 100 ng Plasmid and ddH_2O to a final reaction volume of 20 µl.
The reaction mixtures for all vector samples except pXP322 were incubated at 37 °C for 15 minutes. Vector pXP322 was incubated for 2 hours at 37 °C instead. Xhol was inactivated by incubation at 80 °C for 20 min. Spel was dissociated from the DNA by using a 6x Loading dye containing SDS. After addition of loading dye the reaction mixtures were applied on a 1 % agarose gel. To visualize the DNA, the gel was stained with the Gel Red Nucleic Acid Stain. The gene fragments were then separated from the vector backbones by electrophoresis. The gel was run at 110 Volt for 30 minutes in TAE Buffer. As standard the GeneRuler 1 kb DNA Ladder from Thermo Scientific was used.

4.3.9 DNA extraction from agarose gel

The gel slice containing the DNA fragment of interest was excised from the gel under UV-light using the Gel-Doc 2000 with a scalpel. DNA extraction was performed according to the manufacturer´s instruction manual with the GeneJET Gel Extraction Kit from Thermo Scientific. The isolated DNA fragment was stored at -20 °C.

4.3.10 Cloning of the *M. thermoacetica* genes into yeast shuttle vectors

Genes that were supposed to be transformed into the same strain were ligated into shuttle vectors with different selection markers (LEU2, URA3, HIS3 and MET15) allowing for

transformation of the yeast strain with up to three different expression cassettes. Information about the expression cassettes that were constructed is shown in Table 4.1.

Table 4.1: Information about constructed expression cassettes.

Integration cassette	Vector backbone	Target gene	Selection marker	Selection medium
pXP314-A	pXP314	Moth_1204	Met15	mineral medium containing uracil, L-leucine and L-histidine
pXP318-B	pXP318	Moth_1203	URA3	mineral medium containing L-methionine, L-leucine and L-histidine
pXP320-C	pXP320	Moth_1202	HIS3	mineral medium containing uracil, L-leucine and L-methionine
pXP320-D	pXP320	Moth_1201	HIS3	mineral medium containing uracil, L-leucine and L-methionine
pXP322 -E	pXP322	Moth_1200	LEU2	mineral medium containing uracil, L-methionine and L-histidine
pXP320-F	pXP320	Moth_1199	HIS3	mineral medium containing uracil, L-leucine and L-methionine
pXP318-G	pXP318	Moth_1198	URA3	mineral medium containing L-methionine, L-leucine and L-histidine
pXP320-H	pXP320	Moth_1197	HIS3	mineral medium containing uracil, L-leucine and L-methionine

4.3.11 Sticky-end ligation

The double digested vector backbones pXP314, pXP318, pXP320 and pXP322 were ligated with the double digested DNA fragments containing target genes according to Table 4.1.

The reaction mixture contained 1 µl T4 ligase [5 U/µl], 4 µl 5x DNA ligase buffer, 2 µl 50 % PEG 4000, 25 ng Vector, 75 ng DNA fragment and sterilized ddH_2O to a final volume of 20 µl. As a negative control ddH_2O instead of the insert was used.
The reaction mixtures were incubated for 10 min at 22 °C. After the incubation, 2 µl of each ligation mixture were transformed into 50 µl electrocompetent *E. coli* Top 10 cells. Transformed cells were plated out on LB media containing ampicillin.

4.3.12 Colony PCR

To determine whether the *E. coli* Top 10 colonies obtained after transformation actually contained the appropriate ligated vector constructs (pXP314-A, pXP318-B, pXP320-C,

pXP320-D, pXP322-E, pXP320-F, pXP318-G and pXP320-H), a colony PCR was carried out. The forward primer was designed in order to bind at the TEF1 promoter which was present in all four different shuttle vectors. The reverse primers were designed to bind at the 3' end of the integrated genes. Information about the primers used in this study can be found in Section 3.7, Table 3.5.

The reaction mixture for the colony PCR contained 3 µl 10x DreamTaq buffer, 3 µl dNTPs [2 mM], 1.5 µl forward and 1.5 µl reverse primer [100 µM each], 0.15 µl DreamTaq polymerase [5 U/µl] and 20.85 µl ddH_2O. A single yeast or *E.coli* colony was scratched from the selection medium, transferred to a 0.2 ml PCR tube containing the reaction mixture and resuspended before placing the tubes into the thermocycler.
The PCR program used is shown in Table 4.2.

Table 4.2: Colony PCR. Thermocycler program used for colony PCR.

Cycle step	Temperature [°C]	Time [min]	Cycles
Initial denaturation	95	3	1
Denaturation	95	0.5	
Annealing	58	0.5	30
Extension	72	1	
Final extension	72	5	1
	4	hold	

The PCR products were separated by electrophoresis on a 1 % agarose gel for about 45 minutes using 90 volts. As standard the GeneRuler 1 kb DNA Ladder from Thermo Scientific was used.

4.3.13 Confirmation of correctly isolated expression cassettes

Clones that have been confirmed to harbor the correct assembled expression cassettes by colony PCR were used for the preparation of ONCs, which were further used for isolation of the amplified plasmids as described in Section 4.3.5.
To verify that the correctly ligated expression cassettes were isolated, the obtained plasmids were digested using XhoI and SpeI as described in Section 4.3.6. After the restriction digest the samples were applied to a 1 % agarose gel and examined under UV light.

4.3.14 Construction of linear integration cassettes

Linear expression cassettes were produced with PCR using the constructed expression vectors harboring the target genes see Table 4.1 in Section 4.3.8.

The reaction mixture contained 10 µl 5x Phusion® HF Buffer, 1.5 µl DMSO, 5 µl dNTPs [2 mM], 2.5 µl forward and 2.5 µl reverse Primer [100 µM each], 1 ng template DNA, 0.5 µl Phusion Polymerase [2 U/µl] and ddH_2O to a final reaction volume of 50 µl. For the negative control water was used instead of template DNA. The forward primer used were prepared as to carry around 45 bp of the homologous sequence of the integration site in *S. cerevisiae* S288C at the 5'end followed by the first 20 bp of the TEF1 promoter. The reverse primers were designed to have the loci homologous sequence for integration at the 5'end followed by 20 bp of the antiparallel *loxP* sequence flanking the marker.
Detailed information about the primer can be found in Table 3.6 in Section 3.7. The PCR programs for the different vector constructs are listed in Table 4.3.

Table 4.3: PCR program for the construction of linear expression cassettes.

cooC, CODH, CFeSP small and large, orf7, acsF and mTR				**ACS**		
Cycle step	**Temperature [°C]**	**Time [min]**	**Cycles**	**Temperature [°C]**	**Time [min]**	**Cycles**
Initial denaturation	98	0.5	1	98	0.5	1
Denaturation	98	0.15	30	98	0.15	30
Annealing	80	0.5		90	0.5	
Extension	72	2.5		72	2	
Final extension	72	7.5	1	72	7.5	1

DNA fragments obtained from PCR were applied to a 1% (w/v) agarose gel and separated by electrophoresis for about 45 minutes using 90 volts. As standard the GeneRuler 1 kb DNA Ladder from Thermo Scientific was used. The bands corresponding to the size of the linear expression cassettes were cut out and extracted from the gel as descripted in Section 4.3.7.

4.3.15 Homologous integration of the linear expression cassettes

Selection of integration loci

The integration loci were adopted from a previous paper [58] and should enable stable integration and expression of the target genes in *S. cerevisiae*. Expression cassettes were separately transformed into the corresponding *S. cerevisiae* BY4741 and variant strains. Information about which genes were integrated into which loci are shown in Table 4.4.

Table 4.4: Integration loci in *S. cerevisiae* addressed in this work to integrate the 8 target genes from *M. thermoacetica*.

Expression cassette	Expression cassette length [bp]	Loci	Chromosome	Target gene
YPRCTy1-2-A	3949	YPRCTy1-2	XVI	Moth_1204
YBLMTy1-1-B	4712	YBLMTy1-1	II	Moth_1203
YLRCTy1-1-C	4937	YLRCTy1-1	XII	Moth_1202
TRP1-D	4088	TRP1	IV	Moth_1201
YMLWTy1-1-E	4920	YMLWTy1-1	XIII	Moth_1200
YILWTy3-1-F	3497	YILWTy3-1	IX	Moth_1199
YDRWTy1-5-G	3659	YDRWTy1-5	IV	Moth_1198
YMLWTy1-2-H	3988	YMLWTy1-2	XIII	Moth_1197

The yeast strains were transformed with the linear PCR products and selected by growth on appropriate selection mineral medium. Strains transformed with YPRCTy1-2-A were grown on mineral medium containing uracil, L-leucine and L-histidine. Strains transformed with YBLMTy1-1-B or YDRWTy1-5-G were grown on mineral medium containing L-methionine, L-leucine and uracil. Strains transformed with YLRCTy1-1-C, TRP1-D, YILWTy3-1-F or YMLWTy1-2-H were grown on mineral medium containing L-methionine, L-leucine and L-histidine. Strains transformed with YMLWTy1-1-E were grown on mineral medium containing uracil, L-methionine and L-histidine.

4.3.16 Transformation of *S. cerevisiae* BY4741

Single colonies of *S. cerevisiae* BY4741 were picked to inoculate 50 ml YPD for preparation of an ONC (for more details see Section 4.1.2). Resultant cells were used to inoculate 50 ml YPD medium to a starting OD_{600} of 0.2. They were grown to an OD_{600} between 0.7 – 1.0 before the biomass was harvested by centrifugation for 5 minutes at 5000 rpm and room temperature. The supernatant was removed and the cell pellet was resuspended in 15 ml ddH_2O and centrifuged again for 5 minutes at 5000 rpm and room temperature. The

supernatant was completely removed and the cells were resuspended in 1 ml 100 mM LiAc pH 7.5, transferred to an Eppendorf tube and centrifuged for 15 sec at 13500 rpm at room temperature. The supernatant was removed and the cell pellet was resuspended in 250 µl 100 mM LiAc solution pH 7.5. 50 µl of the pretreated cells were transferred to a new Eppendorf tube and centrifuged for 15 sec at 13500 rpm at room temperature. 350 µl of the transformation mixture containing 240 µl 50 % PEG 3640, 36 µl 1 M LiAc solution pH 7.5, 25 µl Carrier DNA (2 mg/ml single stranded salmon sperm), 1 ng plasmid or linear DNA to be transformed or ddH_2O as negative control were then added, mixed carefully by pipetting up and down and incubated at 30 °C and 350 rpm for 30 min in an Eppendorf tube. Afterwards cells were heat shocked at 42 °C for 20 min at 350 rpm. Cells were separated from the reaction mixture by centrifugation for 15 sec at 13500 rpm at room temperature. The supernatant was removed and the cell pellet was resuspended in 200 µl ddH_2O and plated on the appropriate selection medium. The plates were incubated at 30 °C for 5 days.

Transformed strains were named after the genes that were integrated, with following abbreviations used for the gene names: A for Moth_1204, B for Moth_1203, C for Moth_1202, D for Moth_1201, E for Moth_1200, F for Moth_1199, G for Moth_1198 and H for Moth_1197.

4.3.17 Verification of genomic integration by diagnostic PCR

The genomic integration of the different expression cassettes was verified by colony PCR. The forward primers were designed as to anneal around 50 to 100 base pairs upstream of the integration site. The same reverse primers that were used for the ligation control were taken, annealing at the 3'-end of the according target gene. Information about the primer can be found in Section 3.7 Table 3.7. The PCR programs for the colony PCRs are shown in Table 4.5.

Table 4.5: Diagnostic PCR. PCR program used for verification of genomic integration of the target genes.

Cycle step	Temperature [°C]	Time [min]	Cycles
Initial denaturation	95	10	1
Denaturation	95	0.5	30
Annealing	[a]	0.5	
Extension	72	[b]	
Final extension	72	5	1
	4	hold	

[a] 57.5 °C for cooC (Moth_1204), 59.1 °C for orf7 (Moth_1200), 58.8 °C for CODH (Moth_1203), 60.5 °C for acsF (Moth_1199), 59.4 °C for ACS (Moth_1202), 57 °C for CFeSP small (Moth_1198), 62.8 °C for CFeSP large (Moth_1201) and 60.1 °C for mTR (Moth_1197). [b] 1 minute for cooC (Moth_1204), CFeSP large (Moth_1201) and CFeSP small (Moth_1198), 2 minutes for orf7 (Moth_1200), ACS (Moth_1202) and for CODH (Moth_1203), 1.5 minutes for acsF (Moth_1199) and mTR (Moth_1197).

4.3.18 Marker rescue

Both pBF3038 and pBF3060 plasmids were used for the marker rescue. Before use the plasmids were amplified and isolated as described in Section 4.3.4.

Strain BY4741-AEF, which had the possible maturation proteins orf7, cooC and acsF integrated into the genome, was transformed with pBF3060. Strain BY4741-GHD with the integrated mTR and the small and large CFeSP subunits was transformed with the pBF3038 vector. After the transformation the yeast cells were plated out on mineral medium and incubated at 37 °C for 2-3 days.

The expression of the CreA recombinase was induced by cultivating resultant transformants in YPG medium. YPG was used because the CreA recombinase on pBF3038 and pBF3060 are under the control of a GAL1 promoter which is induced by galactose (see Section 3.6 Table 3.4). To this end single colonies obtained from the transformation (about 5 colonies per transformed strain) were cultivated in 10 ml YPG for 3-6 hours at 30 °C and 350 rpm. 200 transformants cells were individually plated on YPD plates and incubated at 30 °C for 2 days. Obtained cells were replica plated on respective mineral medium selective plates and YPD plates. Cells that have lost all the selection markers and the CreA-expression plasmid were identified by loss of growth on mineral medium plates without uracil, L-methionine, L-leucine and L-histidine.

After the marker rescue the strains were again tested for the integrated genes via colony PCR (see Section 4.3.17), to verify that they had not lost the genes during the marker rescue. The strains that had kept the genes integrated and had lost their selectable markers were used for further strain construction.

4.3.19 RNA purification

The RNA isolation was carried out using the GeneJET RNA Purification Kit from Thermo Scientific following the instructions in the manual.
Single colonies of the yeast strain BY4741, BY4741-AEFB, BY4741-GHD and BY4741-C obtained from ONCs in YPD medium were subjected to RNA isolation. The isolated and purified RNA samples were stored at -80 °C for one week before further use.

4.3.20 Genomic DNA removal from RNA preparations

To avoid false positive results in the qPCR remaining DNA in the RNA preparation was removed. To this end 1 µg RNA were added to an RNase free tube, with 1 µl 10x reaction buffer with $MgCl_2$, 1 µl DNase I[1 U/µl], RNase-free water to 10 µl. The reaction mixture was then incubated at 37 °C for 30 min. Afterwards 1 µl 50 mM EDTA were added and the mixture was incubated at 65 °C for 10 min to inactivate the DNase I.

4.3.21 cDNA synthesis

In the next step cDNA was synthesized from the isolated RNA using the RevertAid H Minus First Strand cDNA Synthesis Kit from Thermo Scientific following the instructions in the manual. 3 µg of DNase digested and undigested RNA from the yeast strains BY4741, BY4741-AEFB, BY4741-GHD and BY4741-C were used for the first strand cDNA synthesis. As negative control a cDNA synthesis sample without reverse transcriptase was used (NTR).
The final reverse transcription reaction products were stored at -80 °C for less than one week until further use.

4.3.22 qPCR

qPCR was performed with an Applied Biosystems 7900HT Fast Real Time PCR System using the QuantiFast SYBR Green PCR kit. Statistical significance of differences in mRNA expression levels was analyzed using the relative expression software tool (REST©, http://www.gene-quantification.de/rest.html) using a pair-wise fixed reallocation test.

The reaction mixture contained 5 µl SYBR Green master mix, 0.2 µl forward and 0.2 µl reverse primer [50µM], 2 µl template DNA and ddH_2O to a final volume of 10 µl.

Products obtained from reverse transcription [150 ng/µl] were used as template. The cDNA sample of the wild type strain was used as a control. The cDNA of strain BY4741-AEFB was used for the verification of Moth_1204, Moth_1203, Moth_1200 and Moth_1199. The cDNA of strain BY4741-GHD was used for Moth_1201, Moth_1198 and Moth_1197 transcripts and the cDNA of strain BY4741-C was used for Moth_1202. The constructed expression cassettes were used as reference genes at a concentration of 1 ng/µl. As a negative control a non-template control (ntc) was applied consisting of the qPCR reaction mixture without RNA or DNA template to check for contaminations by extraneous nucleic acid or primer dimer formation.

The second qPCR run was carried out with the preexisting isolated RNA samples from the BY4741-AEFB, BY4741-C and BY4741-GDH strain at a concentration of 150 ng/µl, and with the prepared no reverse transcriptase control (NTR). The NTR contained all the RT-PCR reagents, except for the reverse transcriptase.

Primers used for the qPCR are depicted in Section 3.7 Table 3.8. The reverse primers were the same as used for the ligation control. The forward primers were designed as to yield PCR products in the range of 90 to 170 bp. The size of the expected resulting PCR fragments is shown in Table 4.6. The qPCR measurements were performed in triplicates.

Table 4.6: qPCR products. Target gene-specific size of the qPCR fragments.

Gene	qPCR Fragment [bp]
Moth_1204	141
Moth_1203	133
Moth_1202	145
Moth_1201	124
Moth_1200	94
Moth_1199	167
Moth_1198	123
Moth_1197	164

4.3.23 Melting curve analysis of the qPCR products

The dye used for qPCR, SYBR green, intercalates unspecific in double stranded DNA. That could be either produced template as well as primer dimers. To determination if the qPCR had produced single, specific products, a melting curve analysis was conducted. Therefore the

thermocycler in which the qPCR was carried out was programmed to carry out the melting curve analysis at the end of the qPCR amplification rounds. For the production of a melting curve the temperature in the thermocycler was continuously increased while the occurring fluorescence resulting from the dye dissociating from the DNA was measured.

4.3.24 Conditioning of the glove box

The glove box was used to provide an oxygen-free environment for the preparation of cell-free extracts and the enzymatic assays, as the recombinant enzymes are highly oxygen sensitive. To establish anaerobic conditions inside the glove box, the box was subjected to the following procedure every time before using it as well as after placing materials inside. The inner atmosphere of the glove box was removed by creating a vacuum, after which the box was filled with N_2. This procedure was repeated 4 times. The same procedure was applied to all material entering the glove box in a separate chamber of the device.

4.3.25 Studying changes in the yeast growth behavior due to media supplementation

The yeast cultivation medium YPD was supplemented with different additives listed in Table 4.7 which may support formation of functional recombinant enzyme and protein expression. The concentrations of the supplements were selected by comparing their concentration in standard *M. thermoacetica* growth medium and recommended concentrations for *S. cerevisiae* [59, 60, 61].

First an aerobic ONC of *S. cerevisiae* BY4741 was prepared in YPD in shake flasks as described in Section 4.1.3. The additives were sterile filtered (cut-off 0.2 µm) and added to 60 ml growth medium. Prior to cultivation, the growth medium containing the supplements was flushed with sterile N_2 for 45 minutes. Anaerobic cultivation was initiated by inoculating the N_2-flushed medium with the ONCs to an OD_{600} of 0.155. To ensure anaerobic conditions, this step was carried out inside a nitrogen filled glove box. The rubber stoppered glass serum bottles were then put in a shaker at 180 rpm and 30 °C. Samples for OD_{600} measurements were taken from the inoculated medium inside the glove box to maintain anaerobic conditions.

In the first approach each supplement was added to the N_2-flushed YPD once at a time to analyze its sole impact on the yeast growth behavior. The concentration of each supplement used is listed in Table 4.7. YPD which was not inoculated was used as a control (blank).

Table 4.7: Additives and their concentration that were used for the first approach of YPD medium supplementation.

Growth media	Trace element	Concentration [mg/l]	Molar concentration [µM]
YPD + Folic acid	Folic acid	10	22.6
YPD + Cobalamin	Vitamin B_{12}	10	7.37
YPD + Cobalt	$CoSO_4x7\ H_2O$	1.8	11.6
YPD + Nickel	$NiCl_2x6\ H_2O$	0.3	0.31
YPD + Iron	$FeSO_4x7\ H_2O$	10	35.9
YPD + Sulfur	L-cysteine	0.3	8250

In a second approach all supplements were added to N_2-flushed YPD and mineral medium (supplemented with the appropriate markers to complement auxotrophies) together at the concentrations listen in Table 4.8 above. Additionally concentrations of $NiCl_2$ were increased to 0.6 and 1 mg/l for experiments with growth medium containing all other trace elements to analyze the impact of the pooled additives on the yeast growth behavior. The concentration of the supplement used are listed in Table 4.8. YPD which was not inoculated was used as a control (blank).

Table 4.8: Additives and their concentration that were used for the first approach of YPD medium supplementation.

Growth media	Trace element	Concentration [mg/l]	Molar concentration [µM]
YPD + Folic acid	Folic acid	10	22.6
YPD + Cobalamin	Vitamin B_{12}	10	7.37
YPD + Cobalt	$CoSO_4x7\ H_2O$	1.8	11.6
YPD + Nickel	$NiCl_2x6\ H_2O$	0.3, 0.6 and 1	0.31, 0.62, 1.03
YPD + Iron	$FeSO_4x7\ H_2O$	10	35.9
YPD + Sulfur	L-cysteine	0.3	8250

4.3.26 Preparation of cell-free extract

Because the recombinant enzymes and proteins are highly oxygen sensitive all working steps were carried out under anaerobic conditions in a Glove box.

For analyzing the activity of the recombinant enzymes, cell-free extracts were prepared from the BY4741, BY4741-AEFB, BY4741-C and BY4741-GHD strains. Therefore aerobic ONCs from the respective strains were prepared, which were used for inoculating 60 ml N_2 flushed YPD supplemented with 10 mg/L folic acid, $FeSO_4x7\ H_2O$ and vitamin B_{12}, 1.8 mg/L $CoCl_2$, 1 g/L L-cysteine and 1 mg/L $NiCl_2$ to an starting OD_{600} of 0.05. The cells were cultivated anaerobically in a shaker at 180 rpm and 30 °C and harvested at an OD_{600} of 0.7 when they were still in the exponential growth phase. The yeast cells were separated from media components by centrifugation at 5000 rpm for 10 minutes at 4 °C. After the supernatant was discharged, the cell pellets obtained were suspended (two times its weight (w/v)) in 50 mM potassium phosphate buffer pH 7.0. The same mass of glass beads as obtained cell pellet was added. The cell pellets were subsequently disrupted by vortexing of the mixture for 45 sec and putting it on ice immediately for another 45 sec 10 times in a row. Then the samples were centrifuged for 10 min at 5000 rpm and 4 °C the supernatant was decanted and put on ice immediately.

The obtained cell-free extracts were stored on ice in the glove box and used for the activity assays immediately. The protein concentration was measured with the Bradford assay.

4.3.27 SDS-PAGE

Cell-free extracts from anaerobically grown wild type yeast or recombinant strains BY4741-AEFBC, BY4741- GHD and BY4741- AEFGHD were used for SDS-PAGE analysis.

In a first approach crude cell-free lysates with a protein content of 1µg were used for the preparation of reduced samples for the SDS-Gel. In a second approach the same amount of cell-free lysate was incubated at 55 °C for 25 minutes in the glove box before preparation of reduced samples. After the heat treatment the cell-free extract was centrifuged at 5000 rpm for 5 minutes and room temperature to remove the precipitated protein.

<u>Preparation of reduced sample</u>

1µg cell-free extract (crude or heat treated), 1 µl Reducing Agent (10x), 2.5 µl LDS Sample Buffer (4x) filled up with deionized water to 10 µl were incubated at 70 °C for 10 minutes for protein denaturation.

SDS-Gel

3 µl standard and 10 µl reduced sample were then loaded onto a 4-12 % Bis-Tris Mini Gel. The upper and lower buffer chambers were filled with the appropriate 1x running buffer. 200 V were applied constantly for 1 hour. As a standard the Thermo Scientific PageRuler prestained protein ladder was used.

Silver staining

The gel was incubated for 20 minutes in a fixation solution. Then a sensitizing solution was added for 20 minutes. The solutions were removed and the gel was washed in H_2O 4 times for 5 minutes. Then the gel was placed in a silver solution for 15 minutes. The solution was removed and the gel was washed in H_2O for 1 minutes after which the developing solution was applied. After visualization of the protein lanes the stopping solution was added in which the gel was incubated for 10 minutes. The developed gel was washed in H_2O and then a picture was taken with a digital camera.

4.4 Enzymatic assays

As the recombinant enzymes and proteins are highly oxygen sensitive all enzymatic assays were conducted under anaerobic condition in a glove box. As the recombinant enzymes have not been expressed in *S. cerevisiae*, the enzymatic assays were adapted from the literature and the assay conditions adjusted.

4.4.1 Determination of specific enzyme activity

Quantification of enzyme activity was done by correlating the change in absorbance with the increase or decrease of substrate concentration over time using the following equation:

$$\frac{\Delta c}{\Delta t} = \left(\frac{Abs_2 - Abs_1}{t_2 - t_1}\right) / \varepsilon * d$$

ε = extinction coefficient or molar absorptivity [$M^{-1}cm^{-1}$]

c = concentration [M]

t = time [min]

d = path length of the sample cuvette [cm]

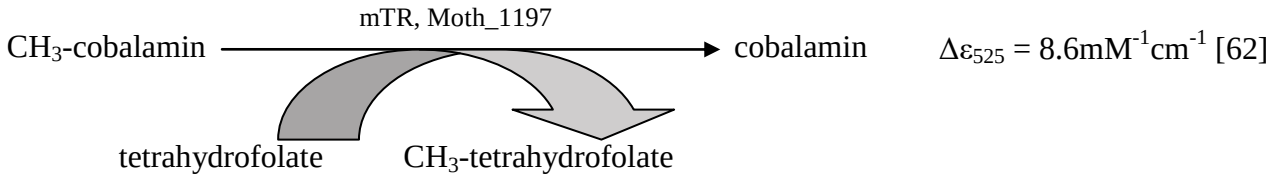
Abs_1 and Abs_2 are the absorptions measured at a certain time point (t_1 and t_2 respectively).

The initial velocity was used for determining the change in absorbance over time [min^{-1}]. Then the concentration per time [M/min] was determined by dividing the change of absorbance over time with the extinction coefficient [$M^{-1}cm^{-1}$] and the thickness of the cuvettes which was 1 cm. This was then multiplied with the dilution factor of the cell-free lysate in the reaction mixture to get the mol/min. The used amount of protein was then used to calculate the specific enzyme activity [mol/min/mg].

4.4.2 mTR assay

To analyze the functional expression of the *M. thermoacetica* methyltransferase, its activity was measured by recording the demethylation of methylcobalamin at 525 nm.

Reaction



Assay conditions

The assay was carried out as described by David Lee Roberts et al. [62].
The reaction mixture contained 50 mM potassium phosphate buffer pH 6.8, 66 µM methyl-vitamin B_{12}, 300 µM tetrahydrofolate, and 5 mM dithiothreitol in a total volume of 0.4 ml.
The mixture was flushed with nitrogen for 15 minutes and then preheated to 55 °C on a thermocycler for 5 minutes. 100 µl freshly prepared cell-free extract were also preheated for 5 minutes at 55 °C and centrifuged at 1350 rpm for 5 minutes at room temperature after which the protein concentration in the supernatant was determined.

The 400 µl preheated assay mixture plus 100 µl supernatant of the preheated and centrifuged cell-free extract with a protein concentration of 12.9 mg/ml for BY4741, 18.7 mg/ml for the BY4741-GDH strain and 13.5 mg/ml for the BY4741-AEFGDH strain (undiluted, 4:1 or 1:2 diluted with nitrogen flushed 50 mM potassium phosphate buffer pH 7.0) were gently mixed

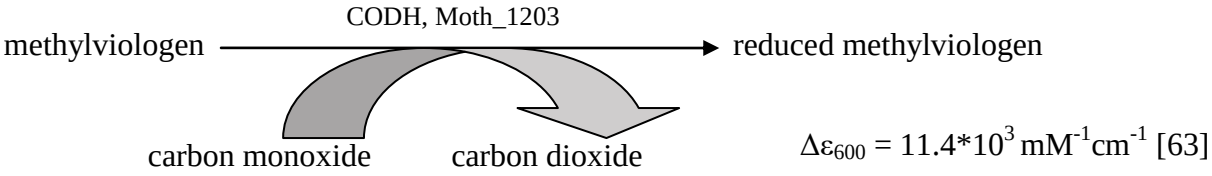
and put on a thermocycler at 55 °C and 150 rpm during the entire duration of the assay (8.75 hours). The reaction mixture plus 100 µl of 50 mM potassium phosphate buffer pH 7.0, which was also flushed with nitrogen for 15 minutes, was used as a reference.

The mTR Assay was done discontinuously. Eppendorf tubes with 2 ml reaction mixture were prepared. For each measurement 0.5 ml of the reaction mixture from the 2 ml tube were transferred into a fresh Eppendorf tube and centrifuged at 1350 rpm for 5 minutes to remove precipitated proteins. The supernatant was transferred into a plastic cuvette and the demethylation of methylcobalamin at 525 nm was measured on a Beckman – Coulter DU 800 UV/Vis Spectrophotometer outside the glove box, as the mTR is not oxygen sensitive. The absorption was measured right after the cell-free extract was added to the assay mixture, 5 minutes after the addition, and then after 1h, 1.75h, 2.75h, 3.75h, 5.75h and after 8.75h.

4.4.3 CODH assay

The carbon monoxide dehydrogenase activity was determined by following the reduction of methylviologen at 600 nm.

Reaction



Assay conditions

The assay was carried out as described by J E Clarks et al. [63].

The assay mixture contained 50 mM potassium phosphate buffer pH 7.4, 30 mM methylviologen, 3.2 mM DTT and ddH_2O in a total volume of 0.4 ml. The reaction mixture was flushed with nitrogen for 45 minutes to remove the oxygen before use. The reaction mixture was then incubated at 55 °C for 5 minutes before the cell-free extract was added.

100 µl freshly prepared cell-free lysate of the BY4741 and the BY4741-AEFBC strain were also preheated for 5 minutes at 55 °C. After the 5 minutes the cell-free lysate was centrifuged at 1350 rpm for 5 minutes. The supernatant was transferred into a new Eppendorf tube and the protein concentration determined. 100 µl of this supernatant were then added to the 0.4 ml

reaction mixture in a serum stoppered cuvette. The reaction mixture plus 100 µl of 50mM potassium phosphate buffer pH 7.0 was used as a reference.
Various approaches were carried out in order to verify and quantify CODH activity in the wild type and the recombinant yeast strains.

i)

5 ml carbon monoxide were slowly sparged into the serum-stoppered cuvettes containing 0.5 ml reaction mixture, shortly after the preheated cell-free lysates were added. The protein concentration of the used cell-free lysates were 0.1 mg/ml. The methylviologen reduction was measured continuously on a Beckman Coulter DU 800 UV/Vis Spectrophotometer over a period of 24 hours.

ii)

The setup of the second approach differed from the first one in the point that all measurements were carried out inside the glove box and that the 5 ml carbon monoxide were added in to the CODH assay mixture before the cell-free extract was added. The protein concentration of the cell-free extracts used were 0.8 mg/ml. The DS 11+ Spectrophotometer from DeNovix was put into the glove box and used for the photometric measurement. The absorption at 600 nm was discontinuously measured after 10, 15, 25, 40, 55 and 70 minutes.

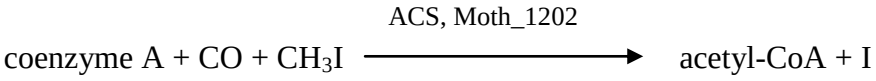
iii)

In this approach at first no carbon monoxide was sparged into the anaerobic assay mixture. The protein concentration of the cell-free extracts used were 0.9 mg/ml. 100 µl preheated cell-free extract were added to 0.4 ml of the anaerobic assay mixture to start the reaction. The cuvettes containing the assay mixture were kept at 55 °C in a water bath and were only removed for the absorption measurements. After the measurements the cuvettes were again placed in the water bath. Measurements were carried out every 5 minutes and the reaction was proceeded for 60 minutes. After one hour the reaction mixture was gently mixed by inverting and then carbon monoxide was sparged slowly into the assay mixture to avoid the formation of foam. Afterwards also the headspace was filled with 5 ml of carbon monoxid and the absorption at 600 nm was measured during another hour. Again the cuvettes containing the assay mixture were kept at 55 °C in a water bath and were only removed for the absorption measurements. After the measurements the cuvettes were again placed in the water bath. Measurements were carried out every 5 minutes for 1 hour.

4.4.4 ACS assay

The ACS activity was measured by the synthesis of acetyl-CoA from iodomethane, carbon monoxide and coenzyme A, in accordance with the method used by Abbanat D. et al. [64].

Reaction



Acetyl-CoA formed was quantified by liquid chromatography coupled to mass spectrometry LC-MS at the Institute for Biomedicine and Health Sciences (HEALTH) at the Joanneum Research Forschungsgesellschaft m. b. H., Graz by Gert Trausinger.

Assay conditions

The ACS assay was conducted using the cell-free extracts from the strain BY4741AEFBC, BY4741C and BY4741. As a control the assay mixture was incubated with 50 mM potassium phosphate buffer pH 7.0 instead of cell-free extract.

The ACS reaction mixture contained 50 mM MES (pH 6.5), 2 mM $MgCl_2$, 2 % (vol/vol) ethylene glycol and 1 mM CoA in a total volume of 80 µl. The assay mixture was flushed with nitrogen for 15 minutes and after that it was sparged with carbon monoxide for 2 minutes.

After that, 100 µl cell-free extract with a protein concentration of 0.8 mg/ml were added to the assay mixture. The reaction was initiated by the addition of 1 µl CH_3I [1M]. After initiation of the reaction the assay mixture was incubated at 55 °C. Samples were taken once before heating, after one hour and after 7 hours. The reaction was stopped by the addition of 100 µl of aerobic ddH_2O and by placement of the mixture on ice followed by a heat treatment at 99 °C for 2 minutes and centrifugation for 10 minutes at 13200 rpm and 4 °C. The supernatant was then frozen at -70 °C before LC-MS analysis. Samples were transported to the institute for biomedicine and health sciences at Joanneum Research where the acetyl-CoA concentrations were determined by LC-MS. Data acquisition was conducted using the Xcalibur software and data interpretation was also carried out at HEALTH.

5 Results

5.1 Strain construction

To construct recombinant yeast strains harboring the different *M. thermoacetica* target genes, a suitable cloning strategy was designed. The genetic work flow that was established is illustrated in Figure 5.1.

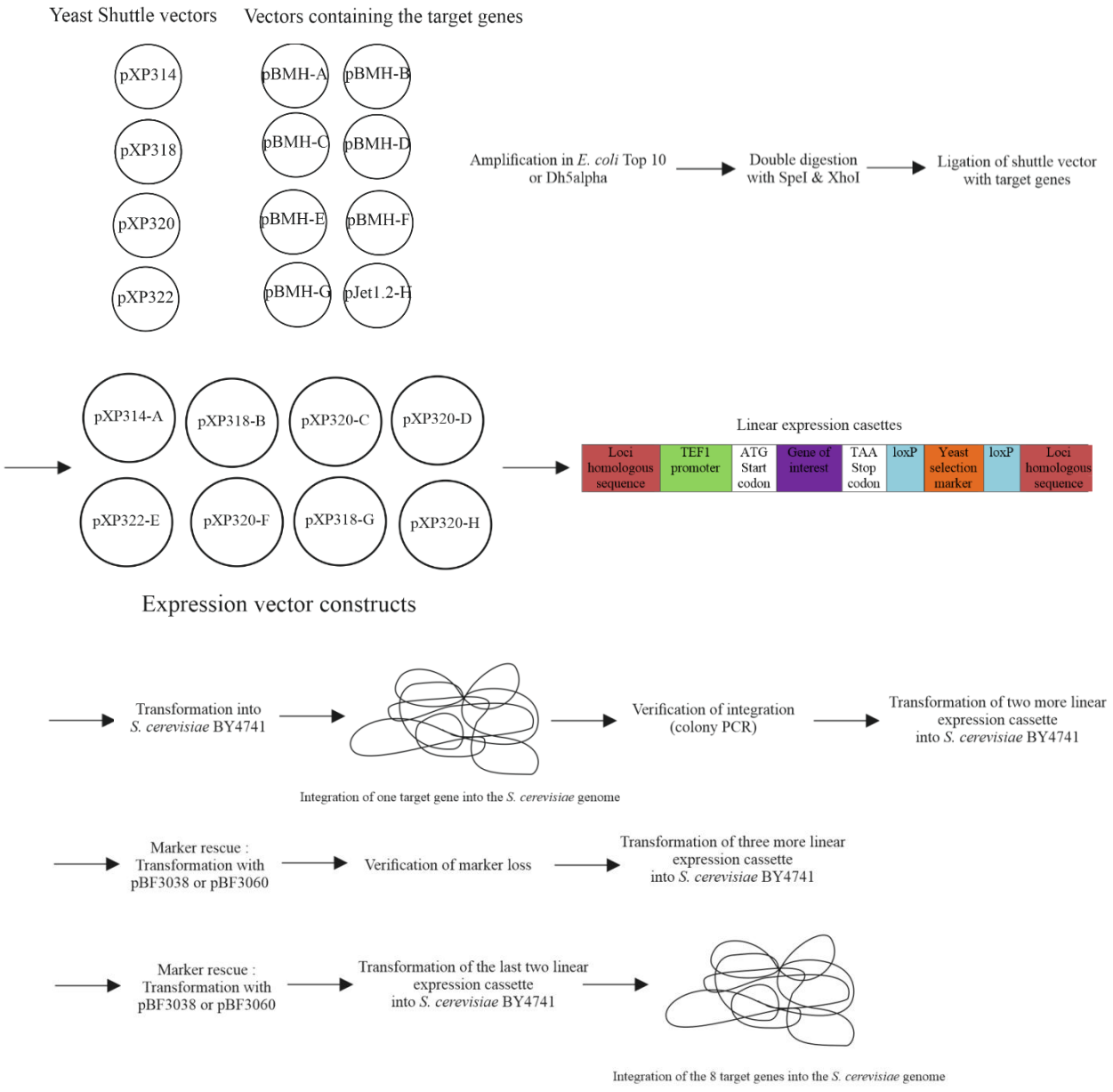


Figure 5.1: Strain construction work flow. Graphical representation of the genetic working steps enabling multiple gene integration into BY4741.

5.1.1 Vector amplification

After the amplification of the shuttle vectors and the vectors containing the target genes in *E. coli* DH5alpha and *E. coli* Top 10 respectively, the plasmids were isolated at a concentration between 100 and 350 ng/µl. Variation of obtained concentrations was not found to be plasmid specific.

5.1.2 Expression cassettes

5.1.2.1 *M. thermoacetica* genes

The respective size of the DNA fragments obtained after successful SpeI and XhoI digestion of the pBMH and pJET1.2 plasmids harboring the target genes were calculated and are listed in Table 5.1. Figure 5.2 shows the successful cut of the eight target genes out of their vector backbones after separation in an agarose gel. DNA bands corresponding to the target genes were cut out and subsequently eluted from the gel, resulting in DNA fragment with a concentration between 10 and 100 ng/µl.

Table 5.1: Information about the size of the 8 target genes from *M. thermoacetica*, and the pBMH and pJET1.2 vector backbone.

Vector	Gene name	Gene size [bp]	Vector backbone size [bp]
pBMH-Gene A	Moth_1204	762	2917
pBMH-Gene B	Moth_1203	2037	2917
pBMH-Gene C	Moth_1202	2202	2917
pBMH-Gene D	Moth_1201	1353	2917
pBMH-Gene E	Moth_1200	1920	2917
pBMH-Gene F	Moth_1199	762	2917
pBMH-Gene G	Moth_1198	984	2917
pJET1.2-Gene H	Moth_1197	801	2984

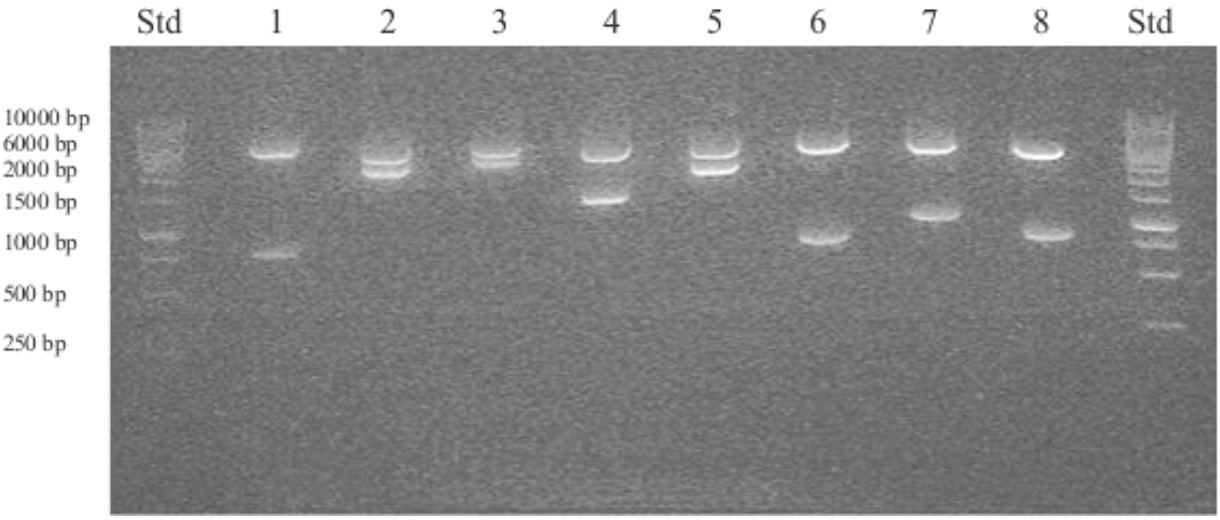


Figure 5.2: Separated DNA fragments after double digestion of the Biomatik vectors containing the target genes from *M. thermoacetica* with SpeI and XhoI. Lane 1 represents the cut pBMH-GeneA vector, lane2 represents the cut pBMH-GeneB vector, lane 3 represents the cut pBMH-GeneC vector, lane 4 represents the cut pBMH-GeneD vector, lane 5 represents the cut pBMH-GeneE vector, lane 6 represents the cut pBMH-GeneF vector, lane 7 represents the cut pBMH-GeneG vector and lane 8 represents the cut pJET1.2-GeneH vector.

5.1.2.2 Shuttle vectors

Successful SpeI and XhoI digestion of the shuttle vectors pXP314, pXP318, pXP320 and pXP322 results in an 18 bp DNA fragment corresponding to the cut out multiple cloning site. This applies for all digested plasmids. The second DNA fragment obtained from pXP314, pXP318, pXP320 and pXP322 digestion results in a 5615bp, 5103 bp, 5163 and 5428 bp long DNA fragment respectively. The digested fragments were separated on an agarose gel and detected under UV light (see Figure 5.3). DNA fragments displaying the expected size were cut out and eluted from the gel (see Material part Section 4.3.9). Resulting DNA-buffer solutions contained the desired DNA fragment at a concentration between 0.01 and 0.1 µg/µl. Because the 18 bp multiple cloning sites are much smaller than the rest of the vectors, their concentration on the gel was not high enough for visualization. Therefore it was not possible to distinguish between single or double digested vectors. For this reason a successful double digest was proven only after successful ligation of shuttle vector backbone and the Spel and XhoI digested target genes.

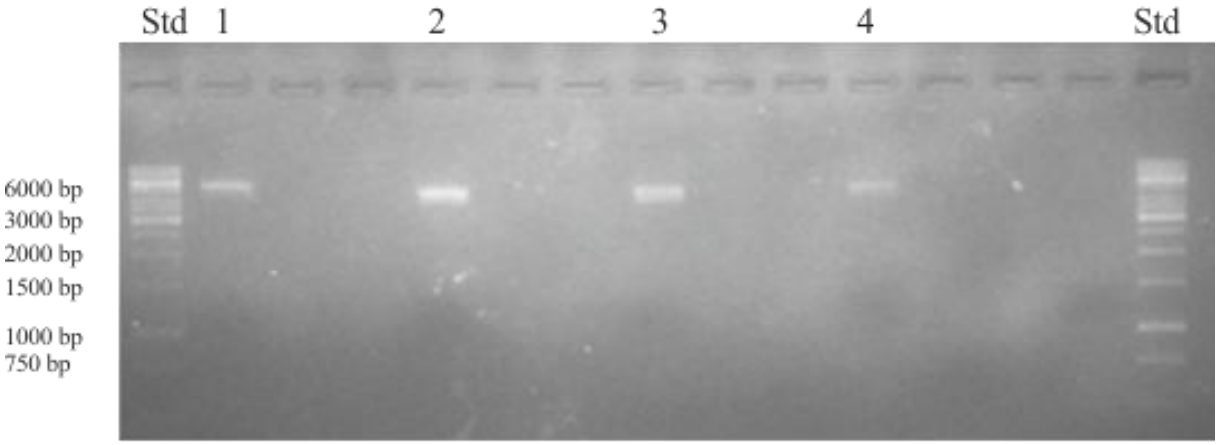


Figure 5.3: Shuttle vector pXP314, pXP318, pXP320 and pXP322 after SpeI and XhoI double digestion. The digested vectors were applied to an agarose gel to separate the vector backbone from the cut out vector piece after double digestion with SpeI and XhoI. Lane 1 represents the cut pXP314 vector, lane 2 represents the cut pXP318 vector, lane 3 represents the cut pXP320 vector and lane 4 represents the cut pXP322 vector.

5.1.2.3 Expression cassettes

The results from the colony PCR of the *E. coli* Top 10 cells which were transformed with the ligated expression vector constructs are shown in Figure 5.4. Successful ligation of the target genes with their shuttle vectors resulted in a PCR product of 1227 bp for vector construct pXP314-A. For pXP318-B a 2502 bp, for pXP320-C a 2667 bp, for pXP320-D a 1818 bp, for pXP322-E a 2385 bp, for pXP320-F a 1227 bp, for pXP318-G a 1449 and for pXP320-H a 1266 bp long PCR fragment should be obtained. Preparation of all 8 expression vectors was confirmed by comparing the calculated size of the PCR products with the size of the obtained DNA lanes in Figure 5.4.

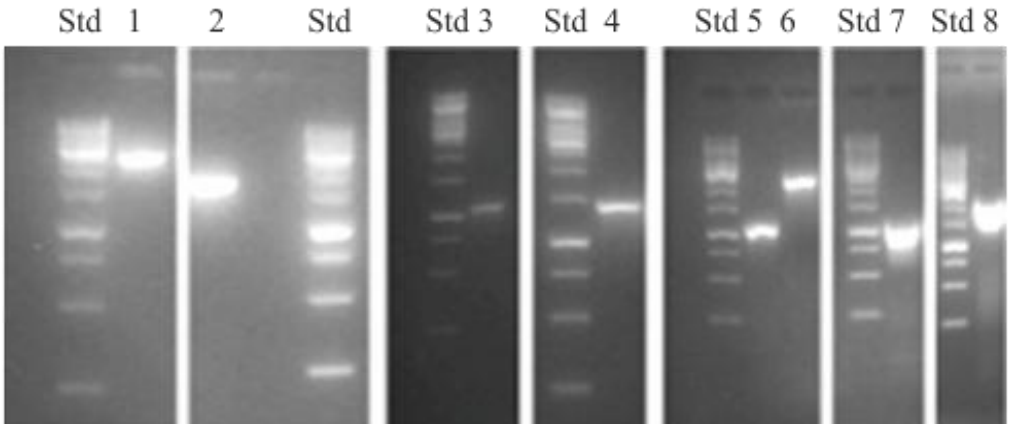


Figure 5.4: Resulting PCR products from the colony PCR conducted to control correct ligation of the expression cassettes. Colony PCR of transformed *E. coli* Top 10 cells harboring the vector constructs pXP314-A (lane 10), pXP318-B (lane 2), pXP320-C (lane 11), pXP320-D (lane 3), pXP322-E (lane 15), pXP320-F (lane 6), pXP318-G (lane 8) and pXP320-H (lane 13). The size of the standard lanes from top to bottom are 10000bp, 8000bp, **6000bp**, 5000bp, 4000bp, 3500bp, **3000bp**, 2500bp, 2000bp, 1500bp, **1000bp**, 750bp, 500bp and 250bp. Bright standard lanes with higher DNA concentration are indicated in bold letters.

After the expression cassettes were amplified in *E. coli* and isolated at a concentration between 10 and 100 ng/µl an additional restriction double digest was conducted to verify that each isolated vector construct carried the intended gene. The corresponding electropherogram is shown in Figure 5.5. Correctly ligated plasmids resulted in one DNA fragment with the size of the shuttle vector and another fragment with the size of the target gene shown in Figure 5.5 (for comparisons see Material part Section 3.6). Comparing sizes of target gene sequences with the experimental obtained electrophoretic mobility of the digested DNA fragments confirmed correct vector construction in 7 out of 8 constructs. In case of pXP322-E no DNA insert could be detected.

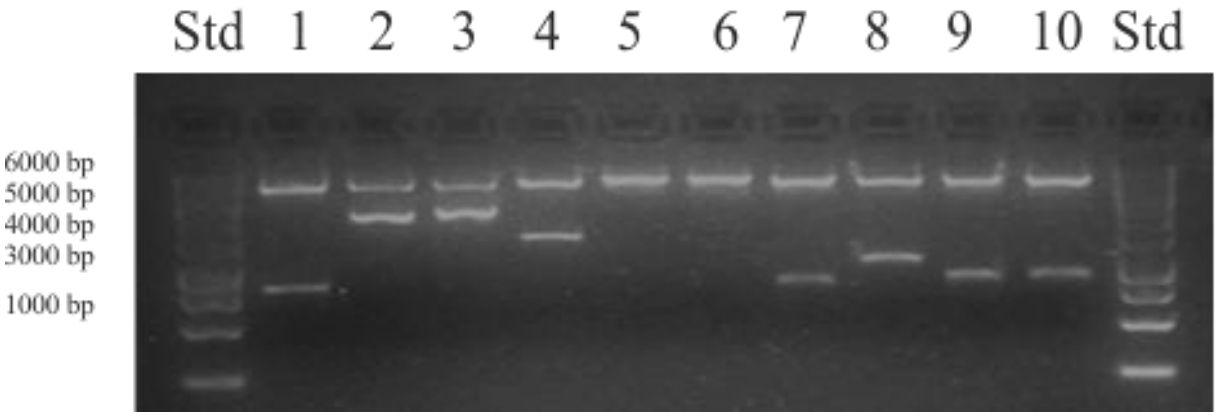


Figure 5.5: Resulting DNA fragments after double digestion of the expression vectors with XhoI and SpeI. Lane 1 is the cut pXP314-A vector, lane 2 corresponds to the cut pXP318-B, lane 3 pXP320-C, lane 4 to pXP320-D, lane 5 and 6 pXP322-E, lane 7 to pXP320-F, lane 8 to pXP318-G and lane 9 and 10 to pXP314-H.

To enable the construction of the pXP322–E expression vector the incubation time for the restriction digest of the pXP322 shuttle vector was altered from 15 minutes to 2 hours, which then resulted in correctly ligated pXP322 –E vector constructs.

5.1.2.4 Construction of linear expression cassettes

The *S. cerevisiae* mechanisms for homologous recombination were used for the integration of the target genes into the host genome. The linear expression cassettes were prepared by PCR using the constructed expression vectors as templates. An illustration of the general composition of the linear cassettes and their genetic features is shown in Figure 5.6.

Loci homologous sequence	TEF1 promoter	ATG Start codon	Gene of interest	TAA Stop codon	*loxP*	Yeast selection marker	*loxP*	Loci homologous sequence

Figure 5.6: General composition of the established linear expression cassettes and their genetic features. The linear expression cassettes used for homologous gene integration in *S. cerevisiae* harbored the gene of interest under the control of a TEF1 promoter, a yeast selection marker flanked by two *loxP* sites, as well as two loci homologous sequences at the 3' and 5' end.

PCR products obtained were applied and separated on a 1 % (w/v) agarose gel. Comparing the calculated length of the expression cassettes (Method part Section 4.3.13) with the obtained PCR fragments shown in Figure 5.7 revealed that all linear expression cassettes displayed the expected size and therefore were prepared successfully. To assure that only the linear expression vectors were cut out and eluted from the gel and further used for yeast transformation, the expression vectors used for their preparation were also applied on the gel for size comparison. The negative control samples of the PCR for the preparation of the linear expression cassettes were also applied on the gel.

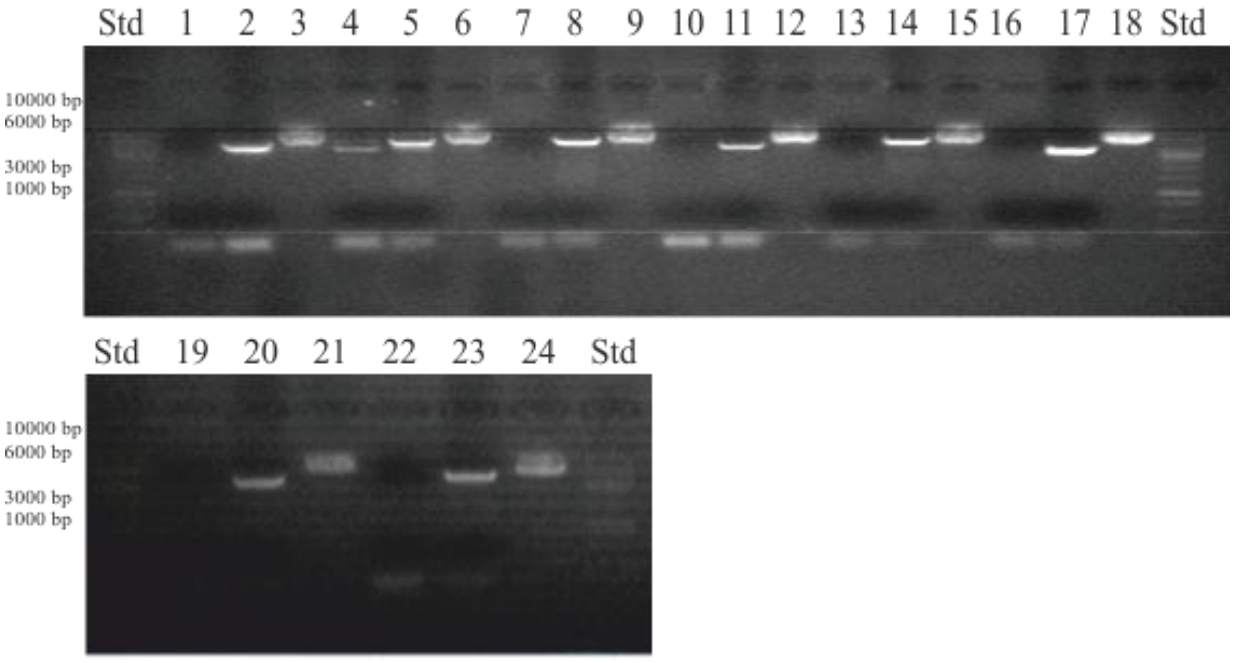


Figure 5.7: Electropherograms of the linear expression cassettes constructed in this work. Concentration of agarose gel: 1 %. Lane 1, 4, 7, 10, 13, 16, 19 and 22 show the results of the negative control samples of the PCR for the preparation of linear expression cassettes for gene cooC, CODH, ACS, CFeSP large, orf7, acsF, CFeSP small and mTR. Lane 2, 5, 8, 11, 14, 17, 20 and 23 show the pXP314-A, pXP318-B, pXP320-C, pXP320-D, pXP322-E, pXP320-F, pXP318-G and pXP314-H expression vectors. Lane 3, 6, 9, 12, 15, 18, 21 and 24 show the linear expression cassette prepared from pXP314-A, pXP318-B, pXP320-C, pXP320-D, pXP322-E, pXP320-F, pXP318-G and pXP314-H.

5.2 Recombinant strains

5.2.1 Verification of transformation and genomic integration

To verify transformation and integration of the target genes at the right chromosomal site, a colony PCR was conducted (Method part, Section 4.3.15). Results from the colony PCR are shown in Figure 5.8 and 5.9. Only if the integration of the linear expression cassettes was successful in the respective yeast strains, PCR fragments of the following size should be obtained. 1401 bp for cooC, 2906 bp for ACS, 1992 bp for the large and 1614 bp for the small CFeSP subunit, 2594 bp for orf7, 1487 bp for acsF and 1443 bp for mTR. From Figure 5.8 it can be seen that the produced PCR products correlate with the calculated sizes for 7 out of 8 genes. Therefore correct genomic integration of all genes except the CODH gene could be verified in the various recombinant strains.

Nevertheless successful transformation of a BY4741 yeast strain with CODH could be verified by colony PCR carried out with the same primer used for the ligation control. As is shown in Figure 5.9 a PCR product with an expected size around 2689 bp for the transformed CODH was obtained.

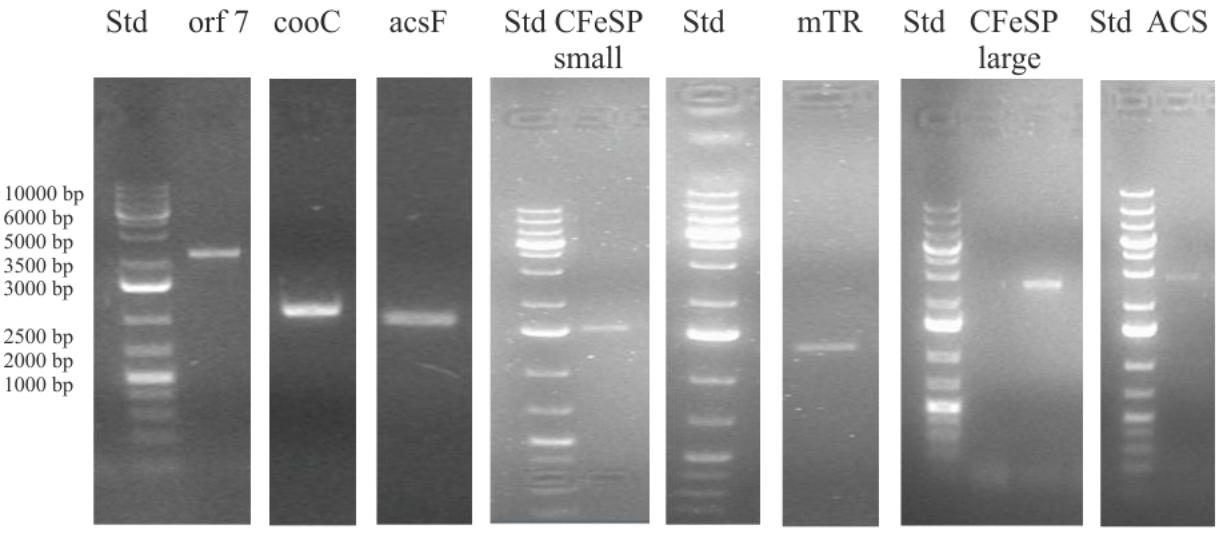


Figure 5.8: Verification of genomic integration. Electropherogram of the PCR products obtained from the colony PCR conducted to verify genomic integration of the *M. thermoacetica* genes orf7, cooC, acsF, the small and large CFeSP subunit, mTR and ACS by colony PCR in *S. cerevisiae* BY4741.

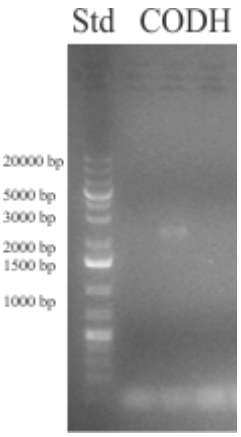


Figure 5.9: Verification of CODH transformation. Electropherogram of the colony PCR product obtained for the verification of the CODH transformation in *S. cerevisiae* BY4741-AEFBC.

5.2.2 Strain constructs

Through transformation of the BY4741 strain with one or several target genes, eight recombinant strain constructs were developed harboring one or more target genes integrated into the yeasts genome at the selected integration loci. The established constructs are listed in Table 5.2. A summary of loci addressed can be found in the Method part Section 4.3.13.

Table 5.2: Constructed recombinant yeast strains. Information about the strain constructs that were produced during this thesis.

Strain construct	Verified integrated target gene	Verified transformed target gene
BY4741-C	ACS	
BY4741-H	mTR	
BY4741-GD	CFeSP small and CFeSP large	
BY4741-GHD	mTR, CFeSP small and CFeSP large	
BY4741-AEF	cooC, orf7, acsF	
BY4741-AEFB	cooC, orf7, acsF	CODH
BY4741-AEFGHD	cooC, orf7, acsF, mTR, CFeSP small and CFeSP large	
BY4741-AEFBC	cooC, orf7, acsF, ACS	CODH

Strain BY4741-C contains the ACS integrated in the yeast genome at chromosome XII, strain BY4741-H harbors the mTR on chromosome XIII and strain BY4741-GD the small and large CFeSP subunits on chromosome IV. Strain BY4741-GHD harbors the small and large CFeSP subunit as well as the mTR integrated in its genome. Strain BY4741-AEF has the helper proteins cooC, orf7 and acsF integrated in its genome on chromosome XVI, XIII and IX. Strain BY4741-AEFGHD carries 6 recombinant genes namely the three helper proteins as

well as the small and large CFeSP subunit and the mTR. Strain BY4741-AEFBC has the ACS, orf7, cooC and acsF stably integrated into its genome. Furthermore the transformation of the CODH could be verified in strain BY4741-AEFBC.

After several cultivation rounds, the established strain constructs were again tested for chromosomal integration of the respective target genes by colony PCR (Method part, Section 4.3.15). PCR fragments with the respective size for each target gene were obtained for all genes except the CODH. Nevertheless even after several rounds of cultivation the CODH DNA sequence was found in strain BY4741-AEFBC. Summarized these results suggest successful production of recombinant yeast strains with stably integrated target genes.

5.2.3 Transcription of the recombinant genes in their host strains

To determine whether the target genes were integrated into active regions of chromatin and transcribed, a qPCR was conducted. The fluorescence signal, formed through intercalation of the fluorescent dye in double stranded DNA, was measured, which corresponds to the amount of double-stranded DNA produced. The higher the template concentration in the sample, the earlier a certain fluorescence threshold is reached. It takes a couple of amplification cycles for the fluorescence signal to cross this threshold. Those numbers of cycles are defined as Ct (cycle threshold) value. As a positive control the expression vectors used for the preparation of the linear expression cassettes were used. The results of the first qPCR run are shown in Table 5.3. A second qPCR run was carried out additionally to add a no reverse transcriptase control. The obtained Ct values are shown in Table 5.4.

In general a difference of 1 in the Ct values indicates a 10 fold higher (-1) or lower (+1) concentration.The Ct values of the PCR products obtained from the transformed strains are significantly lower than the Ct values of the wild type and the ntc displayed in Table 5.3. According to these results it can be concluded that all target genes except Moth_1201, corresponding to the large subunit of the CFeSP, are transcribed in the recombinant yeast strains harboring the respective target genes, but not in the wild type strain. This is because most of the samples show a three order of magnitude concentration difference. Therefore the transcription of all target genes except the large subunit of the CFeSP could be verified using this procedure. The functionality of all designed primer was confirmed by the resulting Ct values for the expression vectors.

The NRT was included to determine whether the removal of the genomic DNA was successful. If a product is seen in the NRT, it probably indicates that contaminating DNA is present in the sample. It is known that it is nearly impossible to remove all genomic DNA from the RNA samples, but by comparing the Ct values of the NRT with the other samples treated with a reverse transcriptase it can be determined if in fact mRNA was detected or just the genomic DNA of the target genes. Because also a product can be seen in the NRT control samples in Table 5.4, it can be assumed that contaminating DNA is present in the prepared RNA samples from strain BY4741-AEFB, BY4741-C and BY4741-GHD. However obtained Ct values for the gene products for all target genes except Moth_1201 were significantly higher in the recombinant strains than in the NRT. According to these findings it can be concluded that mRNA of the respective target genes is present in the transformed BY4741 strains.

Table 5.3: Results of the first qPCR. The wild type and recombinant strains BY4741-AEFB, BY4741-C and BY4741-GHD were used to analyze the transcription of genes Moth_1197 to Moth_1204. Ntc indicates the non-template control.

Gene	Ct			
	***S. cerevisiae* BY4741**	**Expression vectors**	**BY4741-AEFB, BY4741-C and BY4741-GDH strain**	**Ntc**
Moth_1204	31.8	19.7	22.4	38.8
Moth_1203	33.7	24.5	23.8	33.5
Moth_1202	29.6	19.8	30.5	30.2
Moth_1201	35.0	23.9	36.8	36.7
Moth_1200	39.3	26.3	22.9	32.7
Moth_1199	35.7	32.7	21.2	37.5
Moth_1198	34.1	20.4	21.4	35.5
Moth_1197	46.1	18.9	20.0	35.1

Table 5.4: Results from the second qPCR experiment. The recombinant strains BY4741-AEFB, BY4741-C and BY4741-GHD were used to analyze the transcription of genes Moth_1197 to Moth_1204. Ntc refers to the no reverse transcriptase control.

Gene	Ct	
	BY4741-AEFB, BY4741-C and BY4741-GDH	**NRT**
Moth_1204	22.9	26.9
Moth_1203	23.9	26.9
Moth_1202	22.7	26.1
Moth_1200	23.7	27.5
Moth_1199	21.9	26.6
Moth_1198	23.1	26
Moth_1197	21.2	25.5

Additionally a melting curve analysis was conducted after the amplification cycles were completed to assess whether the qPCR assays had produced single, specific products or also non-specific products. This was done because the SYBR green dye used for the qPCR binds to any double-stranded DNA product including primer dimmers or contaminating DNA, resulting in false positive results. Therefore to ensure that only the desired amplicons were detected, a melting curve analysis was carried out. The obtained melting curves for all qPCR products are shown in Table 5.5.

Analysis of the melting curves reveals clearly distinguishable curves for the qPCR products from the wild type strain and the curves from the qPCR products from the reference genes and transformed strains. The melting curves for all genes (except for the Moth_1201 sample) in the transformed strains were the same as the melting curves from the reference genes and they both differed from the obtained melting curves from the wild type strain. Therefore it can be concluded that the desired amplicons were detected and that the qPCR was successful in analyzing the transcription of all target genes except Moth_1201.

Table 5.5: Dissociation curves of the qPCR products. The melting curves of the qPCR products from the wild type (W), recombinant BY4741 strains (S) and the reference genes (P) are depicted.

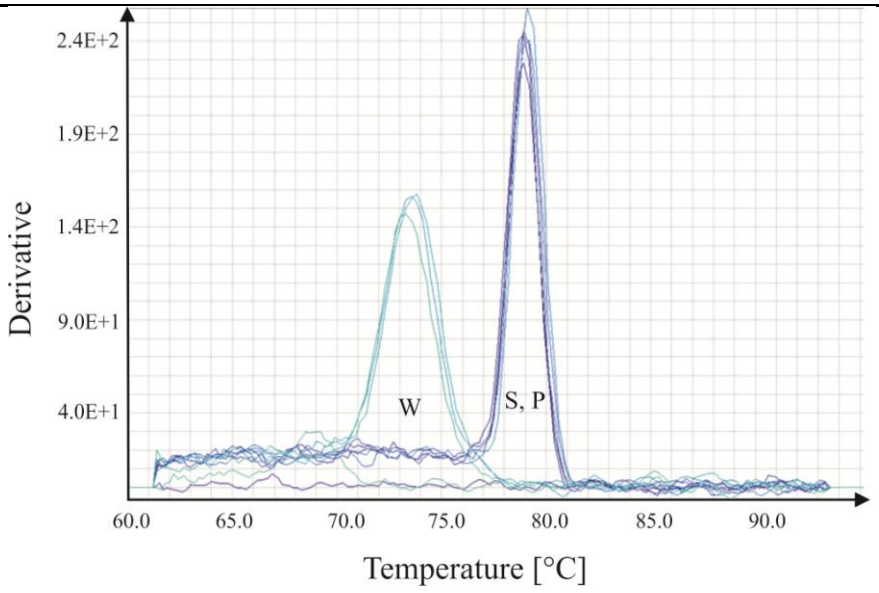
Target Gene	Melting curves
Moth_1204	

Table 5.5: (Continued)

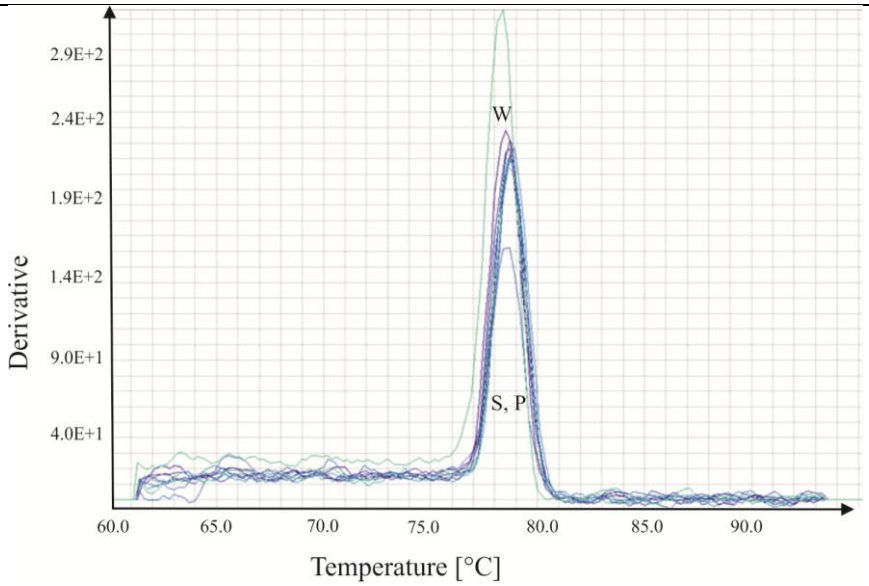
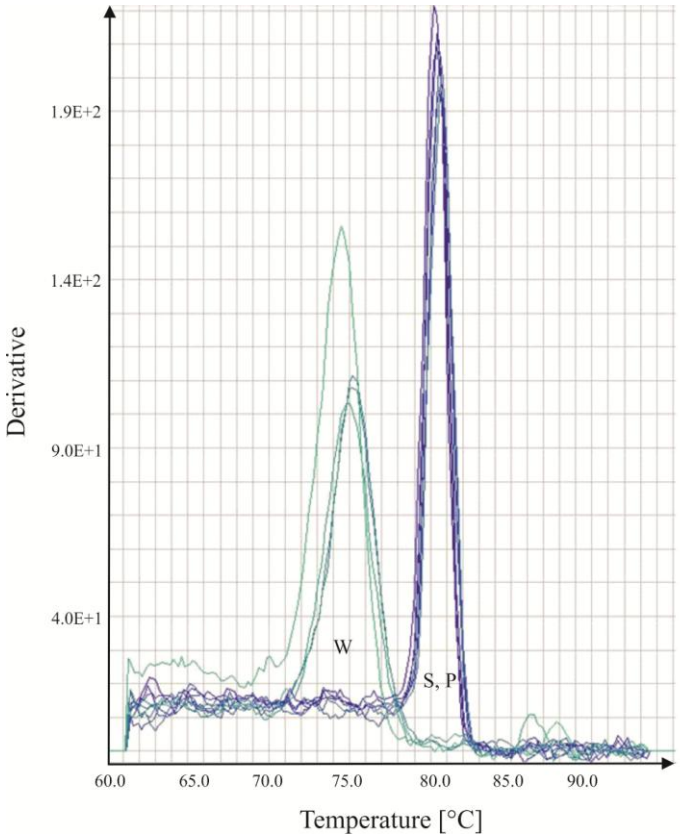
Target Gene	Melting curves
Moth_1203	
Moth_1202	

Table 5.5: (Continued)

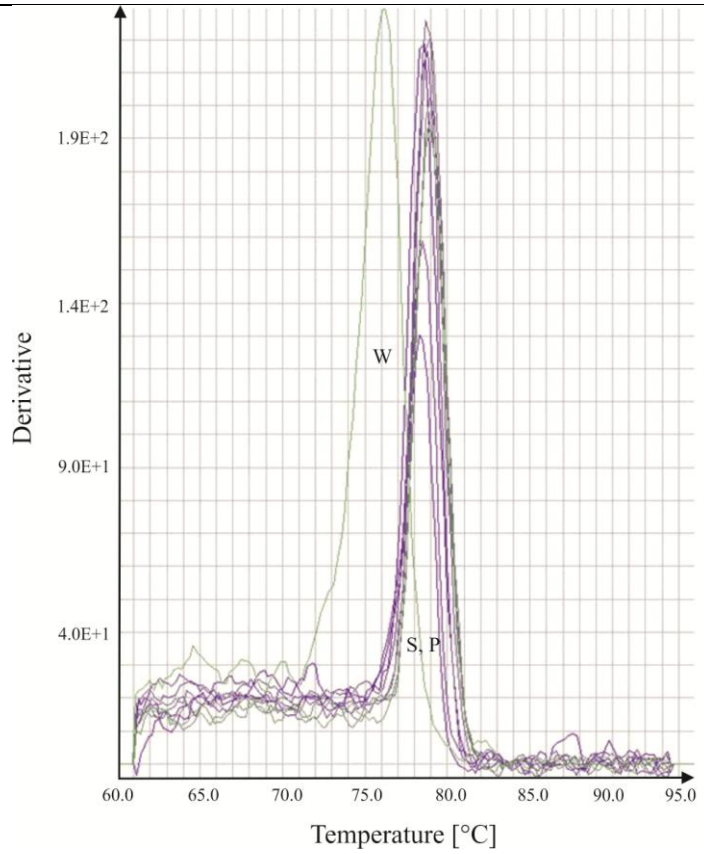
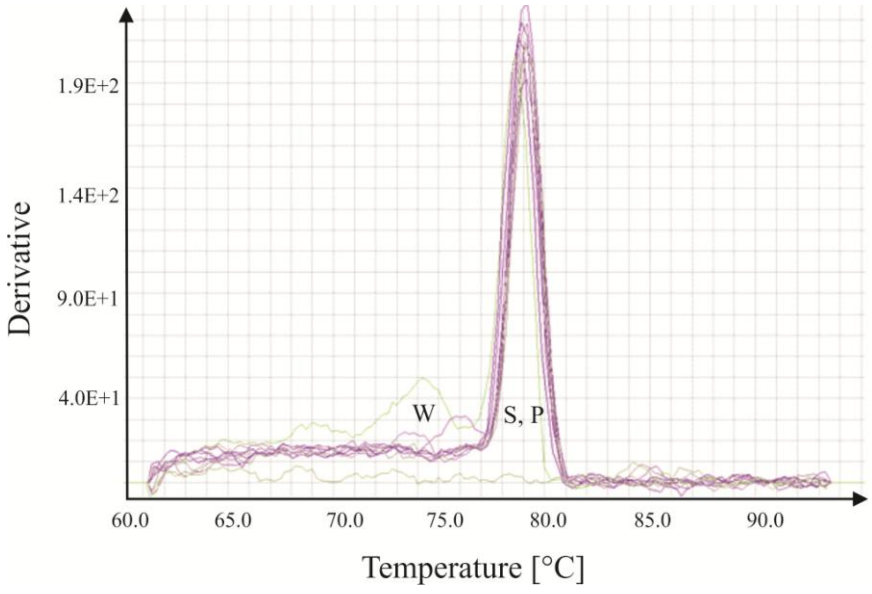
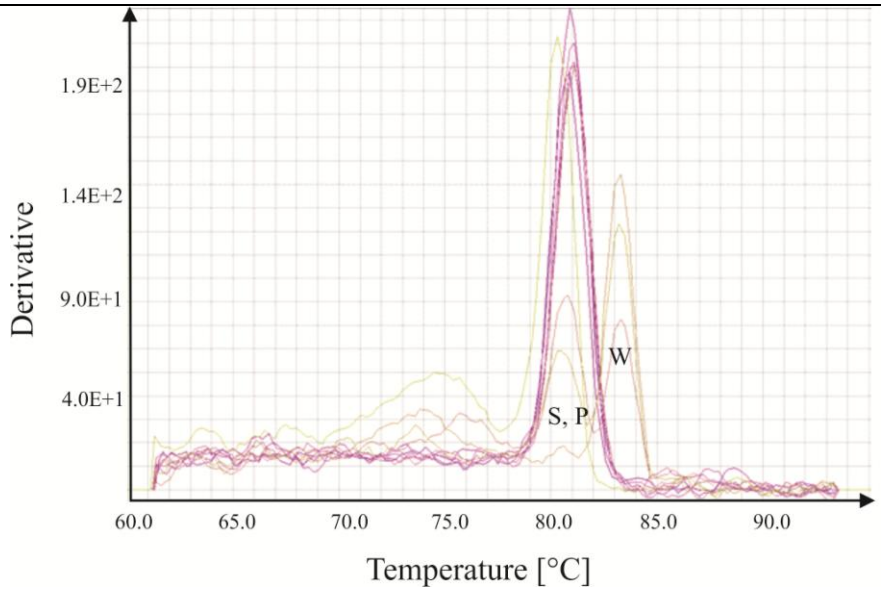
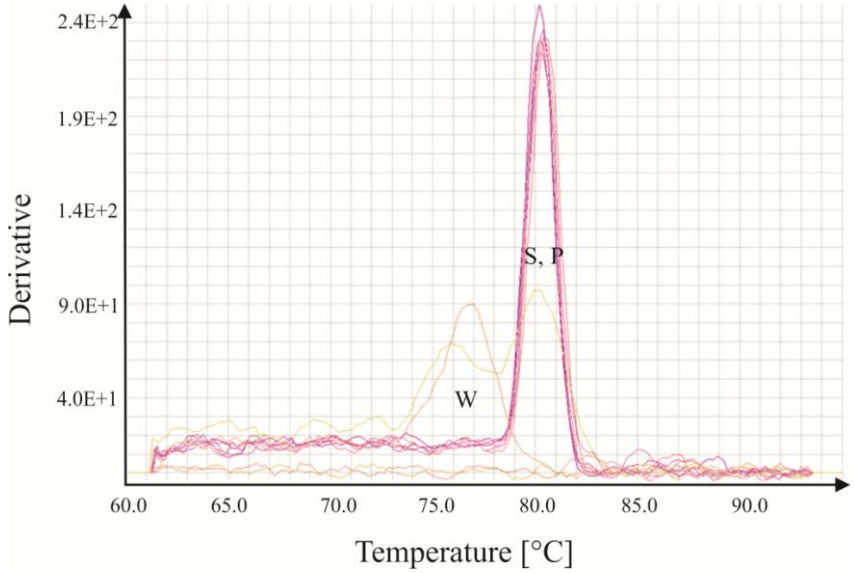
Target Gene	Melting curves
Moth_1200	
Moth_1199	

Table 5.5: (Continued)

Target Gene	Melting curves
Moth_1198	
Moth_1197	

Summarized expression of the target genes in the following combinations (i) CODH, orf7, cooC and acsF, (ii) small CFeSP subunit as well as the mTR and (iii) ACS in the strain constructs BY4741-AEFB, BY4741-GHD and BY4741-C respectively, could be verified by qPCR. The expression of the large CFeSP subunit in strain BY4741-GHD could not be verified.

5.2.4 Expression analysis of the recombinant enzymes and proteins

An attempt to analyze the expression of the recombinant proteins was to separate the proteins from the cell-free extract of the wild type and the recombinant yeast strains through SDS-PAGE. The protein samples that were applied on the gel were either the crude or heat treated cell-free extract of the wild type and recombinant BY4741-AEFBC, BY4741- GHD and BY4741- AEFGHD strains. The protein bands were visualized using a silver staining. Sizes and masses of the recombinant proteins are listed in Table 5.6. The stained SDS gel is shown in Figure 5.10.

Table 5.6: Information about the size and mass of the transformed target genes from M. thermoacetica. Source: http://www.uniprot.org/

Name	Length [bp]	Mass [Da]
Moth_1204	249	26.908
Moth_1203	674	72.924
Moth_1202	729	81.711
Moth_1201	446	48.153
Moth_1200	635	67.466
Moth_1199	249	27.343
Moth_1198	323	35.070
Moth_1197	262	28.609

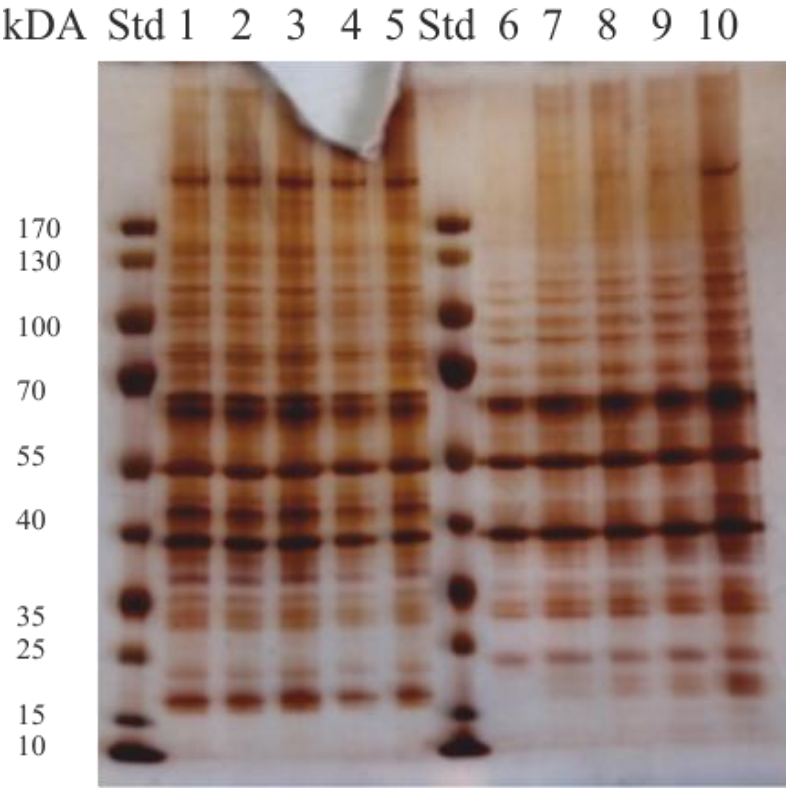


Figure 5.10: SDS-Page of the cell-free extracts from the wild type and recombinant *S. cerevisiae* strains. Cell-free extracts of the wild type yeast strain and the BY4741-C, BY4741-BC, BY4741-GHD and BY4741-AEFGHD strains before (1-5) and after treatment at 55 °C (6-10) were used. Lane 1 and 6 represent the cell-free extract of the wild type BY4741, lane 2 and 7 strain the cell-free extract of BY4741-C, lane 3 and 8 the cell-free extract of BY4741-BC, lane 4 and 9 the cell-free extract of BY4741-GHD and lane 5 and 10 the cell-free extract of BY4741-AEFGHD.

Lanes corresponding to the size of the recombinant enzymes and proteins were compared in the wild type and the transformed strains BY4741-AEFBC, BY4741- GHD and BY4741-AEFGHD. Heat treatment of the cell-free extract sample at 55 °C for 25 minutes resulted in the precipitation of a part of the yeast protein which is indicated in Figure 5.10 by brighter protein lanes in lane 6 to 10 (heat treated) in comparison with lane 1 to 5 (crude cell-free extract).

Neither in the crude nor in the heat treated sample of the BY4741-AEFBC, BY4741-GHD and BY4741-AEFGHD strains an increase of intensity in the lanes corresponding to the size of the target enzymes and proteins could be detected in comparison with the intensity pattern of proteins in the wild type sample.

5.2.5 Growth conditions for functional expression of target genes

The recombinant *M. thermoacetica* enzymes and proteins contain several metal cluster. To enable the nickel and iron-sulfur cluster assembly of the bifunctional CODH/ACS, the helper

proteins acsF, orf7 and cooC were co-expressed and nickel in the form of $NiCl_2$ was added to the growth medium. As high concentrations of nickel are known to be lethal for *S. cerevisiae*, a nickel concentration was determined providing the transformed yeast with appropriate nickel amount required for nickel cluster assembly [65, 66, 67] but not as much as to significantly alter the yeast growth behavior. To provide cobalt and vitamin B_{12} for the CFeSP and the mTR respectively, the growth medium was supplemented with cobalt in the form of $CoCl_2$ and cobalamin. Additionally the growth medium was supplemented with iron in the form of $FeSO_4$ and sulfur in the form of L-cysteine for the iron-sulfur cluster assembly in CODH, ACS and CFeSP.

To analyze how the yeast responded to the altered medium composition, growth curves were recorded for the wild type strain. In a first approach to distinguish the sole effect of each additive and the effect achieved after combining all supplements, the *S. cerevisiae* growth medium YPD was supplemented with each of the additives alone or with a combination of all additive. Results are shown in Figure 5.11. Specific growth rates were determined from the linear part of an $lnOD_{600}$ vs. time plot. They were calculated for each condition and the resultant values are shown in Table 5.7.

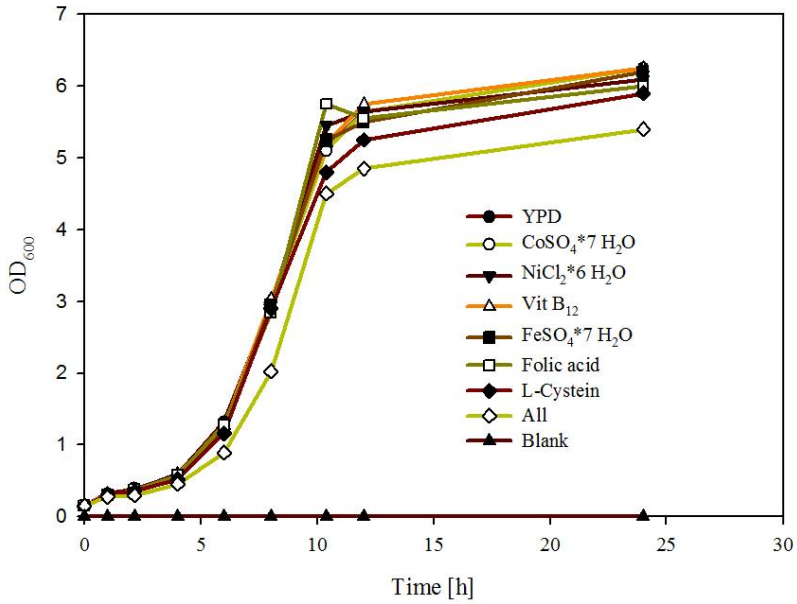


Figure 5.11: Growth behavior of *S. cerevisiae* in YPD supplemented with different trace metals, vitamins and amino acids.

Table 5.7: Specific growth rates of *S. cerevisiae* BY4741 in different supplemented YPD that were determined through linear regression.

	YPD	$CoSO_4$	$NiCl_2$	Vitamin B_{12}	$FeSO_4$	Folic acid	L-cysteine	All additives
μ [h^{-1}]	0.21	0.21	0.23	0.20	0.22	0.26	0.19	0.30

Whether alone or in combination, the additives had no negative influence on the growth rate of the BY4741 strain as shown in Figure 5.11 and Table 5.7. The specific growth rate of the wild type strain in YPD medium supplemented with 22.6 µM folic acid, 1.26 µM $NiCl_2$ or with all pooled supplements was shown to be even higher in comparison with the strains growth in regular YPD medium. Nevertheless the final biomass formation was found to be decreased according to the measured OD_{600} values for yeast grown in YPD supplemented with the pooled additives.

In a second approach all supplements again were pooled and added to YPD. The composition differed only in the $NiCl_2$ concentration from the pooled supplement mix in the first approach. This was done to determine whether the nickel concentration could further be increased without significantly changing the yeasts growth behavior. Either 0.3, 0.6 or 1 mg/l $NiCl_2$ were applied to test the effect of medium supplementation with higher nickel concentrations. Results are shown in Figure 5.12. The resultant values for the specific growth rates are shown in Table 5.8.

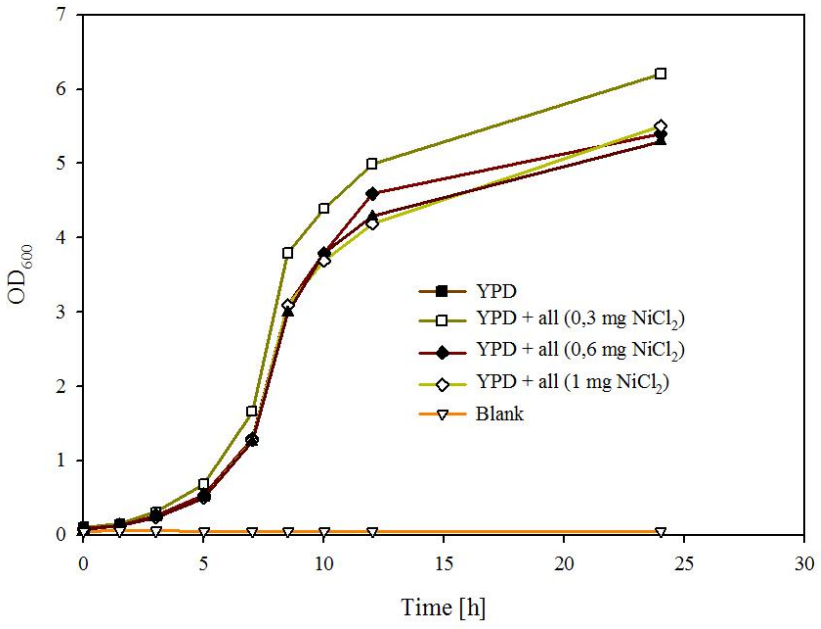


Figure 5.12: Growth behavior of *S. cerevisiae* in YPD supplemented with different trace metals, vitamins and amino acids.

Table 5.8: Specific growth rates of *S. cerevisiae* BY4741 in different supplemented YPD that were determined through linear regression.

	YPD	supplemented YPD [0.31 µM $NiCl_2$]	supplemented YPD [0.62 µM $NiCl_2$]	supplemented YPD [1.03 µM $NiCl_2$]
μ [h^{-1}]	0.55	0.58	0.59	0.58

According to the resulting specific growth rates in Table 5.8, supplementation of YPD with a mixture of 22.6 µM folic acid, 7.37 µM cobalamin, 11.61 µM cobalt, 35.9 µM iron, 8.25 mM sulfur and nickel up to a concentration of 1mg/l had no negative impact on the yeast's growth rate. The specific growth rate for the wild type strain in all supplemented was even slightly increased. Media supplementation with 0.31 µM $NiCl_2$ even resulted in an increase in biomass formation in the second approach. However medium supplementation with 0.62 and 1.03 µM $NiCl_2$ resulted in a decreased final biomass yield of the wild type strain grown in comparison with its growth in regular YPD. But as a maximum yield of biomass was not important for the activity determination of the recombinant enzymes, wild type and recombinant yeast strains used for the preparation of cell-free extracts were grown in YPD media supplemented with 22.6 µM folic acid, 35.9 µM $FeSO_4$, 7.37 µM vitamin B_{12}, 11.61 µM $CoCl_2$, 8.25 mM L-cysteine and 1.03 µM $NiCl_2$.

5.3 Analysis of enzyme activity

To analyze whether the CODH, ACS and mTR from *M. thermoacetica* could be functionally expressed by *S. cerevisiae*, their activity was measured in cell-free extracts. To evaluate the extent of enzyme activity expressed specific activities were determined by relating volumetric activity to the protein content. Because the bifunctional CODH/ACS and mTR represented novel targets for the expression in *S. cerevisiae*, protocols used for activity determination were taken from the literature and adapted.

5.3.1 mTR assay

mTR activity was measured by recording the demethylation of methyl-B_{12} by tetrahydrofolate to form B_{12} and CH_3-H_4folate at 525 nm in the cell-free extract of the wild type BY4741, BY4741-GDH and the BY4741-AEFGDH strain.. The specific mTR activities are shown in Figure 5.13. They are summarized in Table 5.9.

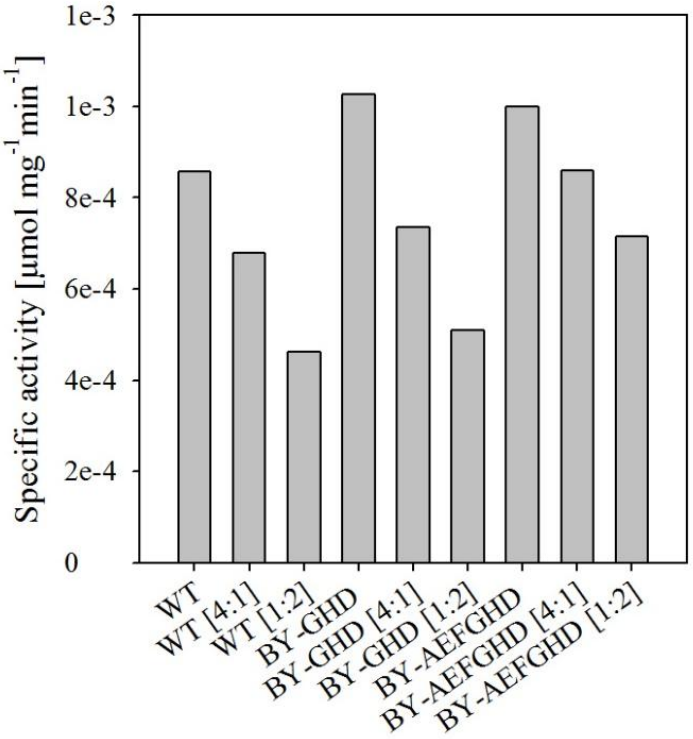


Figure 5.13: Results of the methyltransferase assay. Graphical representation of the specific methyltransferase activities in the undiluted, 4:1 or 1:2 diluted wild type BY4741, BY4741-GHD and BY4741-AEFGHD cell-free extracts.

Table 5.9: Specific mTR activities. The specific methyltransferase activities [µmol mg^{-1} min^{-1}] were determined in the undiluted, 4:1 or 1:2 diluted wild type, BY4741-GHD and BY4741-AEFGHD cell-free extracts.

Strain	Specific enzymatic activity [µmol mg−1 min−1]
Wild type	8.6 x10^{-4}
Wild type [4:1]	6.8 x10^{-4}
Wild type [1:2]	4.6 x10^{-4}
BY4741-GHD	10.3 x10^{-4}
BY4741-GHD [4:1]	7.4 x10^{-4}
BY4741-GHD [1:2]	5.1 x10^{-4}
BY4741-AEFGHD	10.0 x10^{-4}
BY4741-AEFGHD [4:1]	8.6 x10^{-4}
BY4741-AEFGHD [1:2]	7.1 x10^{-4}

A demethylation of methylcobalamin at 525 nm was observed in the cell-free lysate from the wild type (with no recombinant mTR) and both recombinant yeast strains. A 1.19 and 1.16 higher mTR activity was measured in the cell-free extract of the BY4741-GHD and strain BY4741-AEFGHD respectively in comparison with the wild type strain as shown in Table 5.9. According to these results it can be assumed that the recombinant mTR is expressed in an

active form in strain BY4741-GHD and BY4741-AEFGHD but at low expression rates or low activity. Expression of the maturation proteins orf7, cooC and acsF did not seem to have an impact on mTR activity.

5.3.2 CODH assay

Several experimental setups have been attempted in order to analyze the functional expression of the *M. thermoacetica* CODH in *S. cerevisiae* BY4741.

The first approach described in Section 5.3.2 (i) included carbon monoxide bubbling in the assay mixture which already contained the preheated cell-free extract. A picture with the airtight glass cuvettes containing the CODH assay mixture and the cell-free extract 24 hours after starting the reaction is shown in Figure 5.14. Reduction of methylviologen indicated by a color shift from invisible to blue was only observed in the cuvette containing the cell-free lysate from the recombinant BY4741-AEFBC strain which was transformed with the CODH, indicating CODH activity. Though no homogeneous color development was observed in the assay mixture. This prohibited correct quantification of substrate reduction via spectrophotometric measurement. The inhomogeneous color formation might be due to methylviologen oxidation through oxygen entering the cuvettes. To address this problem the second CODH assay was conducted under anaerobic conditions.
Another problem that occurred with this experimental setup was that CO sparging into the reaction mixture for starting the reaction resulted in the formation of foam that remained over a longer period of time and disturbed the photometric measurements.

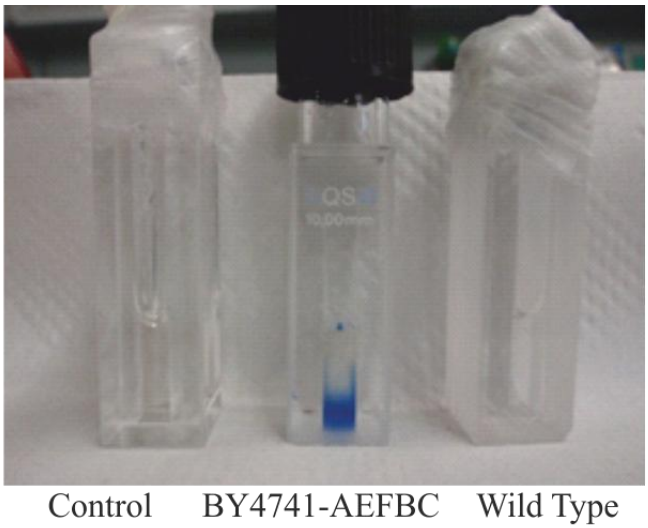


Figure 5.14: Results from the first CODH assay. Cuvettes containing the CODH reaction mixture and the cell-free extract from the wild type and strain BY4741-AEFBC or lysate buffer after 24 hours of incubation at room temperature.

To provide an oxygen-free environment, the second CODH assay was (ii) was carried out inside the glove box. To avoid foam formation by carbon monoxide, this time the prepared assay mixtures were sparged with 5 ml carbon monoxide before the cell-free extract was added.

Reaction mixtures were incubated at room temperature and absorption was measured in regular time intervals. After each measurement a picture was taken with a digital camera. They are shown in Figure 5.15.

As can be seen in the pictures in Figure 5.15, a homogenous color shift from invisible to blue was observed in the CODH reaction mixture containing the cell-free extract from the wild type and BY4741-AEFBC strain. Therefore conduction of the assay inside the glove box seemed to solve the problem of inhomogeneous color formation that was observed in the first experimental setup. No color shift was observed in the negative control.

Nevertheless the color change in the sample containing the cell-free extract from the BY4741-AEFBC was less visible in comparison with the wild type sample indicating a higher methylviologen reduction in the wild type cell-free extract in comparison with the CODH transformed strain. Unfortunately also with this experimental setup the substrate reduction could not be quantified via spectrophotometric measurement. The resulting values were not measured in the linear range of the photometer. The measured data also showed a decrease in absorption at 600 nm instead of the expected increase. Wrong adjustment of the photometer parameter may be the reason therefore. Unfortunately this problem could not be addressed

and no other available photometer at the institute was small enough to fit in the glove box. Therefore the next CODH assay again was conducted outside the glove box.

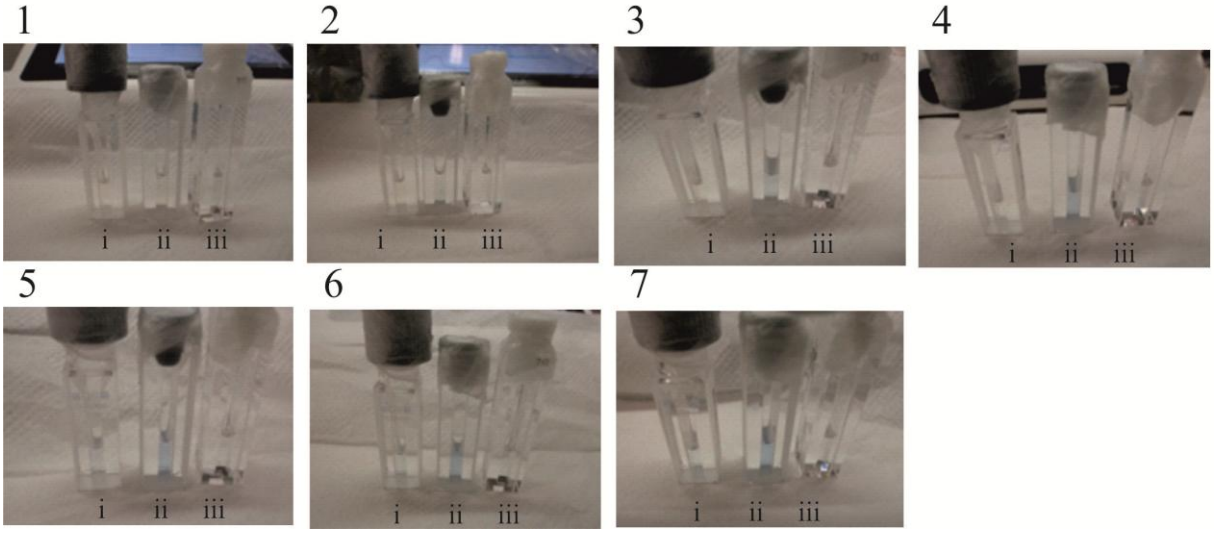


Figure 5.15: Results of the second CODH assay. Pictures of the cuvettes containing the CODH reaction mixture and the cell-free extract of strain BY4741-AEFBC (i), the wild type (ii) and the negative control (iii) before (1) and 10 (2), 15 (3), 25 (4), 40 (5), 55 (6) and 70 (7) minutes after induction.

In the second CODH assay methylviologen reduction was also observed in the cell-free extract of the wild type strain, not harboring the CODH. To analyze CODH unspecific methylviologen reduction, the CODH assay was conducted again but without CO sparging into the reaction mixture. The photometric measurements were again conducted outside the glove box. After one hour of incubation again an inhomogeneous color shift was observed in the reaction mixtures with the cell-free extracts from the wild type and the recombinant BY4741-AEFBC strain. No color shift was observed in the negative control. Due to the observed inhomogeneous color shift a photometric measurement of methylviologen reduction was not possible. Nevertheless the color shift from invisible to blue after one hour of incubation was captured and is shown in Figure 5.16. The results indicate CODH unspecific methylviologen reduction, as the CODH from *M. thermoacetica* needs carbon monoxide for facilitating the reduction of methylviologen.

Inverting the cuvettes containing the assay mixture once, immediately resulted in methylviologen oxidation indicated by a color shift back to transparent. To avoid methylviologen reoxidation by oxygen entering the cuvettes, the headspace of the cuvettes was then filled with 5 ml of CO. This should further enable carbon monoxide transportation into the assay mix during the assay. After one hour of incubation again an inhomogeneous

color shift was observed in the reaction mixtures with the cell-free extracts from the wild type and the recombinant BY4741-AEFBC strain. No color shift was observed in the negative control. Due to the inhomogeneous color shift, the methylviologen reduction could not be quantified. But again a picture was taken from the reaction mixtures after a one hour incubation time for optical verification of methylviologen reduction. The picture is shown in Figure 5.16.

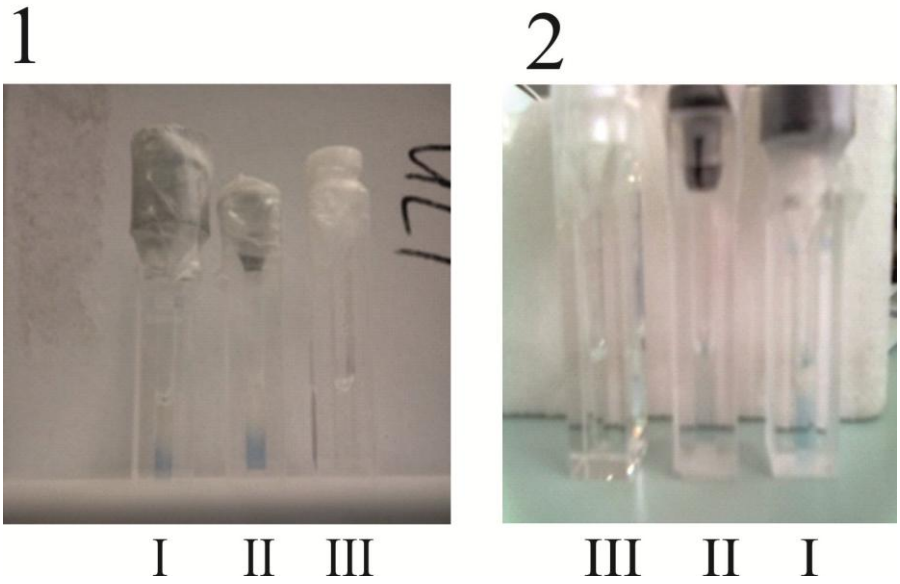


Figure 5.16: Results of the third CODH assay. The cuvettes containing the cell-free extract from strain BY4741-AEFBC (i), the wild type (ii) and the negative control (iii) before (1) and (2) after induction of the CODH with carbon monoxide.

The results of the first CODH assay strongly suggest functional expression of the recombinant CODH in strain BY4741-AEFBC. Nevertheless the results of the second and the third CODH assay show that also CODH independent methylviologen reduction, catalyzed by compounds of the wild type strain, occurs. Therefore a color shift from invisible to blue is not a clear indication of CODH activity. Due to problems with inhomogeneous color changes in the reaction mixtures, no quantification of methylviologen reduction could be achieved. Therefore the activity of the recombinant CODH could not be unequivocally verified.

5.3.3 ACS assay

No acetyl-CoA was formed from iodomethane, carbon monoxide and coenzyme A evidenced by LC-MS measurement of the ACS assay mixture.

6 Discussion

6.1 Cloning of the selected enzymes and proteins

6.1.1 Multiple cloning strategy

A suitable cloning strategy, based on the work of F. Fang et al. [58], was developed allowing stable integration of 7 different heterologous genes from *M. thermoacetica* into *S. cerevisiae*. In this work chromosomal integration of the target genes was chosen over using plasmid-based expression because plasmid based expression systems usually show higher genetic instability [68]. Chromosomal integration on the other hand has been shown to be suitable for multiple gene integration [69].

6.1.1.1 Promoter

To enable high expression levels of the recombinant enzymes and proteins, the strong, constitutive glucose promoter TEF1 (encoding transcriptional elongation factor EF-1α [70]) which has been shown to enable relative constant expression of recombinant proteins in yeast grown on glucose [71, 72], has been used in this work. TEF1 was also chosen because for the production of bioethanol, the recombinant strains are supposed to be grown on glucose, requiring the use of a promoter which is not repressed by glucose, such as TEF1.

6.1.1.2 Selected integration loci in *S. cerevisiae*

The chromosomal integration site is another factor that affects the level of protein expression. F. Fang et al. identified 12 different integration loci that enabled similar expression levels and stable integration. 8 of them were chosen for the integration of the selected *M. thermoacetica* genes [58].

All target genes, except the CODH, were successfully integrated into the chosen loci in the respective recombinant yeast strains as verified by colony PCR (Section 5.2.2 Figure 5.2).
Reasons why the transformation but not the integration of the CODH in strain BY4741-AEFBC could be confirmed include 1) ectopic integration of the gene, 2) failed integration or 3) wrong design of the primer used for verification of genomic integration.

A BLAST analysis of the DNA sequences on chromosome II (where YBLMTy1-1 is located) with the loci homologous sequences on the linear expression cassettes IIYBLMTy1-1-B was carried out. The comparison revealed that various sequences highly homologous to the YBLMTy1-1 integration loci are present on the same chromosome. These might has led to integration of the linear expression cassette at one of the sites sharing high homology to the originally chosen integration site. In this case verification of genomic integration would not be possible with the primers used, as the forward primer was designed to anneal specific to the upstream region of the YBLMTy1-1 loci sequence. Moreover the lack of proof could also be due to incorrect primer design resulting in the formation of primer dimers, secondary structures or incompatible melting temperatures. Therefore it cannot be excluded that the CODH was actually integrated at the YBLMTy1-1 loci or at another site in the host genome. But to address this issue, the colony PCR needs to be repeated using newly designed primers.

Nevertheless the chosen integration sites and the use of the strong constitutive TEF1 promoter, enabled transcription of the integrated target genes, except Moth_1201, as shown by qPCR (see Section 5.2.3).

6.2 Recombinant *S. cerevisiae* strains

The yeast deletion strain BY4741, who already had been used for the integration of multiple genes before, was selected as host strain [58]. Concurrent transformation with up to 4 different expression cassettes was possible using this strain. Employing the bacteriophage *loxP*-CreA system allowed simultaneous removal of the selective markers. This allowed transformation of the recombinant strain again with up to 4 different expression cassettes. Using this strategy, various recombinant yeast strains were established with one harboring up to 6 different target genes integrated into the yeasts genome.

To verify whether genetic manipulation resulted in stable constructs, the recombinant strains were tested for chromosomal integration of the target genes after several cultivation rounds (see Methods part Section 4.3.18). Results provided strong evidence that the target genes, whose genomic integration had been verified, had been stably integrated into the host genome.

6.2.1 Recombinant expression of enzymes and proteins constituting the Wood-Ljungdahl pathway in *S. cerevisiae*

This study is the first attempt to express the enzymes and proteins constituting the Western branch of the Wood-Ljungdahl pathway from *M. thermoacetica* in *S. cerevisiae*. To analyze whether the chosen cloning strategy enabled expression of the recombinant enzymes and proteins, the cell-free extracts of the wild type and the recombinant strains BY4741-AEFBC, BY4741- GHD and BY4741- AEFGHD were separated according to their size in an SDS gel. Unfortunately expression of the selected proteins and enzymes could not be detected using this method, although the expression systems used the strong TEF1 promoter, known to enable high gene expression [72]. Even after heat treatment to precipitate yeast protein, no difference in expression levels in protein lanes corresponding to the size of the target enzymes and proteins could be detected, maybe due to low abundance of soluble protein forms. Conduction of a Western Blot analysis of the cell-free extract represents another possibility to analyze whether the *M. thermoacetica* genes are expressed in *S. cerevisiae* or not. Antibodies for the CODH/ACS, mTR and CFeSP are available [73].
Nevertheless in this work because the expression could not be verified by SDS-Page, the transcription of the target genes was analyzed by qPCR. Transcripts of all target genes except Moth_1201 were found in the respective recombinant strains, indicating transcription of the genes. Although no transcript of the recombinant large CFeSP subunit was detected, the results does not exclude that the gene was transcribed. The primer used for the preparation of the cDNA might not have worked for this protein. This problem could be addressed with the design of gene specific primers. However wrong design of the primer used for the qPCR can be excluded as cause for the negative result, due to the Ct values obtained using the expression vector pBMH-Gene D (harboring Moth_1201) as template (see Table 5.1, Section 5.1.2.1). Another reason why no mRNA of Moth_1201 was detected could be that the site on the chromosome, where the gene was integrated, is not accessible for the transcription machinery. Or maybe the respective mRNA has a short half-life, preventing it to be detected. The mRNA sequence of Moth_1201 was therefore analyzed for degradation sequences using the software RegRNA 2.0. Nevertheless analysis did not reveal mRNA degradation elements (AU-rich elements).

Redesign of the linear expression cassettes containing the CODH and the large CFeSP subunit, with primers who contain sequences complementary to upstream and downstream

chromosomal elements of new integration loci, might enable integration and transcription of both genes. Because some of the integration sites identified by Fang et al. have not been used in this work, for example YDRCTy1-1 or YBLMTy1-5 [58], primer with sequences complementary to upstream and downstream chromosomal elements of those integration loci might be used.

6.3 Activity verification of the selected enzymes and proteins

6.3.1 Recombinant expression of vitamin B_{12} dependent enzymes in *S. cerevisiae*

Functional expression of the recombinant, cobalamin dependent mTR was observed in strain BY4741-AEFGHD and BY4741-GHD grown in YPD that was supplemented with 7.37 µM cobalamin. Although the detected specific mTR activities were considerably low in comparison with the measured specific mTR activity in *M. thermoacetica* (3 µmol min^{-1} mg^{-1}) and in recombinant *E.coli* (13µmol min^{-1} mg^{-1}) [62].

Comparing the results of the mTR assay in Section 5.3.1 Figure 5.13 for the wild type and both recombinant strains, it can be seen that demethylation of the substrate in the mTR assay was caused to a large part by yeast methyltransferases themselves [74, 75]. Furthermore because the assay was conducted inside the glove box, which complicated sample drawing, samples for the measurements could not be taken very often. Conducting the assay outside the glove box, enabling a more frequent sample drawing, might enable a more precise determination of the mTR activity. Also isolation of the recombinant enzyme and its further use for the activity assay instead of using the whole cell-free extract pose a possibility for better characterization of the enzymes activity [62].

Higher activity rates of the recombinant mTR might be achieved by using a different promoter. Because the TEF1 promoter on the pXP vector used is flanked by restriction sites [58], exchanging it with a different promoter, for example the strong glucose promoter PGK1 (encoding phosphoglycerate kinase [72]), is feasible.

6.3.2 Recombinant expression of enzymes containing iron-sulfur cluster in *S. cerevisiae*

Recombinant expression of iron-sulfur cluster proteins such as isopropylmalate isomerase, encoded by *leuC* and *leuD* has already been successfully performed in *S. cerevisiae* by co-expression of genes of the *E. coli* ISC assembly machinery [52]. Nevertheless in this work

functional expression of none of the target genes containing iron-sulfur cluster (CODH, ACS and CFeSP) could be verified. Neither supplementation of the yeast growth medium with iron and cysteine to enhance the probability of iron-sulfur cluster assembly, nor co-expression of the potential maturation proteins acsF, cooC and orf7 did change these results. If the inactivity of the enzymes is associated with improper iron-sulfur cluster assembly, there are other approaches that during this master thesis have not been, but may be tried to enhance iron-sulfur cluster assembly. Those include the elimination of iron-sulfur cluster enzymes in the host yeast [76] or co-expression of protein-specific maturation proteins [52].

6.3.3 Recombinant expression of nickel containing enzymes in *S. cerevisiae*

Research groups have tried to clone and functionally express the bifunctional CODH/ACS, from *M. thermoacetica* in various microorganisms including *E. coli* and recombinant Clostridia [77, 78]. The ACS α subunit for example has already been expressed in an active form in *E. coli* [47]. For the expression in *S. cerevisiae* nevertheless it represents a new target. Genomic integration of the recombinant CODH could not be verified in strain BY4741-AEFB and BY4741-AEFBC. Nevertheless qPCR analysis of isolated RNA from strain BY4741-AEFB, which was used for the preparation of strain BY4741-AEFBC, revealed that CODH mRNA was present. As strain BY4741-AEFBC was exposed to various rounds of cultivation in YPD, lacking selection pressure, but did not lose the CODH gene as verified by colony PCR, it was further grown in YPD and used for analyzation of CODH activity. Nevertheless neither activity of the recombinant CODH nor of the recombinant ACS could be detected, even though the recombinant yeast strain was grown in YPD supplemented with 1.03 µM $NiCl_2$ to enhance nickel cluster assembly in CODH and ACS. Also co-expression of the possible maturation proteins cooC, orf7 and acsF with the CODH and ACS did not have an impact on these results. In comparison, a specific activity of pure CODH from *M. thermoacetica* of about 600 µmol min^{-1} mg^{-1} has been reported [79]. Though the results does not exclude that the enzymes are functionally expressed in the recombinant yeast strains. There are various possibilities why no ACS and CODH activity could be detected. Their specific activities might be too low to be detected with the methods used. Using isolated CODH or ACS instead of the cell-free extract for the activity measurements might represent an approach for further enzymatic assays. This could further be useful to overcome methylviologen reduction by yeast enzymes.

Reasons why the enzymes might not be functionally expressed include incorrect folding followed by subsequent degradation [80], a problem with the codon usage and aggregation or formation of inclusion bodies (which nevertheless is rather unlikely as the enzymes are not over expressed). To address functional expression of the ACS and CODH further studies are required. If the inactivity of the enzymes is associated with improper nickel cluster assembly, nickel concentrations could further be increased to about 5.3 mM $NiCl_2$ [81]. Furthermore the nickel resistance of *S. cerevisiae* could be increased by upregulation of the genes GTT1 and GTT2. Both genes are related to the iron-metabolism and metal homeostasis and have been shown, amongst others, to be upregulated in a nickel-hyper resistant *S. cerevisiae* strain [81].

6.4 Outlook

Since the work for this master thesis was the starting point for the project, further experiments are necessary to address whether the CODH, ACS and CFeSP can be functionally expressed in the constructed yeast stains. If it is not possible to construct a yeast strain functionally expressing the CODH, ACS, mTR and CFeSP, a different cloning strategy might be used. N. B Jensen et al. for example established a vector set, the EasyClone integrative plasmid set [82], which is based on the integrative plasmids from Mikkelsen et al., which has already been used for the successful expression of several genes constituting an indole glucosinolate biosynthetic pathway in *S. cerevisiae* [69]. After developing a recombinant yeast strain that functionally expresses the bifunctional CODH/ACS, mTR and CFeSP, the next step in linking CO_2 fixation with ethanol biosynthesis would be the functional expression of PFOR or ACDH and additional establishment of hydrogenase based regeneration systems. After this the ability of the recombinant enzymes regarding CO_2 fixation and balancing of the redox cofactors needs to be explored.

7 Literature

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8 Appendix

8.1 *M. Thermoacetica* genes optimized for *S. cerevisiae*

DNA sequence of the *M. thermoacetica* genes after optimization for the expression in *S. cerevisiae* as well as the corresponding amino acid sequence and the UniProtKB accession numbers are listed below. Additional restriction sites at the 5’ (SpeI)) and 3’ (XhoI) end of the genes that were added to the DNA sequence are indicated in lowercase letters.

8.1.1 Moth_1204 (762bp), UniProtKB - Q2RJ71

actagtATGAAGTTGGCTATTTCTGGTAAGGGTGGTGTTGGTAAAACTACTATTGCAGCTGGA
TTGATAAAGTATTTCGCTGGTCAAGGTTATCAAGTTTATGCTGTTGATGCTGATCCAGATA
CTTCTTTGGGTATGGTTTTGGGTTTGCCAGAAGAAAGGTTGGGTACTTTGAAGCCTATCGT
TGATATGAGACAAATTATCGCTGAAAGAACTGGTGGTGAGGGTGCTTTTTTCGCTTTGAA
TCCTGAGGTCGATTCATTGTTGGAAGACTTTACTATAAAGAATGATAACATTTTGTTCTTG
AAGATGGGTGCCATTAAGCCAGGTGGTTCTACTTGTTATTGTAGAGAAAATACTGTCTTA
AATGCTATGATAAATTCTTTGTTGTTGAAAAGAAGAGAAATGGTTGTTTTAGATATGGGA
GCAGGTATTGAACATTTAACAAGAGGAACAGCTAGAGGTGTTGATACTATGTTGATTGTT
ACTGAGCCTACTTTAGTTTCTATCCAGACTGCTAGAGTTATTCAAAAATTGGCTTCAGAAT
TGGGAATTGAGCAGATCAAATTCATAGGAAACAAGTTGAGACATCATAGAGATGAAGAG
TTCATCTTAAACCACTTGCCATCTGGTGAAGTTATTGGTTTGATTCCATTTCAACCAGCCA
TTTTGGATCAAGCTGCTGGAATTACTTCTACTCAATCATTTATTGCAACTGGTATTACTGA
ATTAGCCTCaAGATTGATTGAAAATTAActcgag

MKLAISGKGGVGKTTIAAGLIKYFAGQGYQVYAVDADPDTSLGMVLGLPEERLGTLKPIVDM
RQIIAERTGGEGAFFALNPEVDSLLEDFTIKNDNILFLKMGAIKPGGSTCYCRENTVLNAMINS
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NKLRHHRDEEFILNHLPSGEVIGLIPFQPAILDQAAGITSTQSFIATGITELASRLIEN

8.1.2 Moth_1203 (2037bp), UniProtKB - Q2RJ72

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MANDLGVDTPKVPFVASAPEAMSGKAAAIGTWWVSLGVPTHVGTMPPVEGSDLIYSILTQIA
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8.1.3 Moth_1202 (2202bp), UniProtKB - Q2RJ73

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8.1.4 Moth_1201 (1353bp), UniProtKB - Q2RJ74

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VAFARANLAS

8.1.5 Moth_1200 (1920bp), UniProtKB - Q2RJ75

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AVFAGIQSLLKTVSLEIDAIERIIIAGGFGNYLHIPNAVEIGLLPDLPPEKYIFAGNTSLKGAELA
LLSQPAWQETLELARRMTYLELSAGNLFMEEFVSALFLPHTRLELFPSVGNGSGDERRSG

8.1.6 Moth_1199 (762bp), UniProtKB - Q2RJ76

actagtATGGCTAGGCATATTGCAGTTGCTGGTAAAGGTGGTACTGGTAAGACTACTTTTGCT
GCTTTGATGATTAGATATTTGATTGAAGGTCAAAAAGGTTCTATTTTGGCTGTTGATGCTG
ATCCAAATGCTAATTTGAATGAAGCCTTGGGTGTTCAAATCGATACTGTTATTGCTGATAT
CTTGGACGCTACTAAAAATCCAAAGTCTATTCCAGAAGGTATGTCTAAAGAGATCTTCGT
CCAATATCAATTGGCTCAAGCaTTGGTTGAAACTAAAGATTTTGATTTATTGACTATGGGT
AGACCACAAGGTCCAGGTTGCTATTGTTACCCAAATGACTTGTTGAGAAAACACTTGGAA
ACTTTGTCAGATAACTATGACTATATGATTATTGATTCTGAAGCTGGTTTGGAACATATTT
CAAGAAGGATTATTCAGAACGTTTCAGATTTGTTTGTTATTTCTGATGCTTCTGCTAGAGG
TATTAGATCTGCCGGTAGAGTTAGAGAATTGGTACAGGAGTTGCAATTACCTATTAACAA
CTTGTACTTAATAGTTACCAAAACTACTGGTGATATCGCTCCATTGCAAGAAGAAATTGA
AAGAACTGGTATTCCATTGACTGGAGTAATTCCATATGATGAACAGATTGTTGATTATGA
TATTCATTCTAAACCATTGTTTGACTTGCCAGCTACTTCTGTTTCTGTACAAGCTGTTAAA
GCTATTTTAGCTAGATGTCAATTTTAActcgag

MARHIAVAGKGGTGKTTFAALMIRYLIEGQKGSILAVDADPNANLNEALGVQIDTVIADILDA
TKNPKSIPEGMSKEIFVQYQLAQALVETKDFDLLTMGRPQGPGCYCYPNDLLRKHLETLSDN
YDYMIIDSEAGLEHISRRIIQNVSDLFVISDASARGIRSAGRVRELVQELQLPINNLYLIVTKTTG
DIAPLQEEIERTGIPLTGVIPYDEQIVDYDIHSKPLFDLPATSVSVQAVKAILARCQF

8.1.7 Moth_1198 (984bp), UniProtKB - Q2RJ77

actagtATGGCTGTCCAGATTTTGAGAGATAGGTCTAGGGCTGCTGTTCAGAAAGTTGTTTTA
GGTGCTACTAAAGATCAAGGTGGTACTAGATCTCATACTATTGTCGTAGGTGGTGATGCA
GCTTTGCCATTTCATCACTTTGAAGGTGAGATCGTTAACAGACCAGTCATAGGTATGGAA
GTTCAAGATATTGTTCCTGACTGGCCTGATGTTTTGAAAGATCCATTTACAGATGTTATTA
ACGAACCAGGTAGATGGGCTCAAAAGTGTGTAGCTGAATATGGAGCTGATTTGATTTACT
TGAAATTGGACGGTGCTGACCCAGAGGGTGCCAATCATTCTGTTGACCAGTGCGTTGCTA
CTGTTAAAGAAGTTTTGCAAGCTGTTGGTGTTCCATTGGTTGTAGTTGGATGTGGAGATGT
CGAAAAAGACCATGAGGTTTTAGAGGCTGTTGCTGAAGCTGCCGCTGGTGAAAACTTGTT
ATTGGGAAATGCAGAACAAGAGAACTATAAGTCTTTGACTGCTGCTTGTATGGTTCATAA
ACATAACATTATTGCCAGGTCACCTTTGGATATTAACATTTGTAAACAATTGAACATTTTG
ATTAATGAGATGAATTTACCATTGGACCACATTGTAATTGATCCATCTATTGGTGGTTTGG
GTTACGGTATTGAGTATTCATTTTCTATTATGGAAAGAATTAGATTGGGTGCTTTGCAGGG
TGATAAGATGTTGTCTATGCCAGTTATTTGTACTGTAGGTTATGAAGCaTGGAGAGCTAAA
GAGGCTTCTGCTCCAGTTTCAGAATATCCTGGATGGGGTAAGGAAACTGAAAGAGGTATC
TTGTGGGAAGCTGTAACTGCTACCGCTTTGTTACAGGCTGGTGCTCATATTTTGTTGATGA
GACATCCTGAAGCAGTTGCTAGAGTTAAGGAGAATATTGATCAGTTGATGGTATCTAATG
CTTATTAActcgag

MAVQILRDRSRAAVQKVVLGATKDQGGTRSHTIVVGGDAALPFHHFEGEIVNRPVIGMEVQ
DIVPDWPDVLKDPFTDVINEPGRWAQKCVAEYGADLIYLKLDGADPEGANHSVDQCVATVK
EVLQAVGVPLVVVGCGDVEKDHEVLEAVAEAAAGENLLLGNAEQENYKSLTAACMVHKH
NIIARSPLDINICKQLNILINEMNLPLDHIVIDPSIGGLGYGIEYSFSIMERIRLGALQGDKMLSMP
VICTVGYEAWRAKEASAPVSEYPGWGKETERGILWEAVTATALLQAGAHILLMRHPEAVAR
VKENIDQLMVSNAY

8.1.8 Moth_1197 (801bp), UniProtKB - Q2RJ78

actagtATGTTGATTATTGGTGAAAGGATTAATGGTATGTTTGGTGATATTAAGAGAGCAATA
CAAGAAAGAGATCCAGCTCCAGTCCAAGAATGGGCTAGAAGACAAGAAGAAGGTGGTGC
TAGAGCTTTAGACTTGAATGTAGGTCCAGCTGTTCAAGATAAAGTATCTGCTATGGAGTG
GTTGGTTGAAGTTACTCAAGAAGTTTCTAATTTGACATTGTGTTTAGATTCTACTAACATT
AAAGCTATTGAAGCTGGTTTGAAAAAATGTAAGAACAGAGCTATGATTAACTCAACTAAT
GCTGAAAGGGAGAAAGTCGAAAAGTTGTTTCCTTTGGCTGTTGAACATGGTGCTGCTTTG
ATCGGTTTGACTATGAATAAAACAGGTATTCCAAAAGATTCTGATACTAGATTGGCATTT
GCCATGGAATTGGTTGCAGCCGCTGACGAATTTGGATTGCCAATGGAAGATTTGTATATT
GATCCATTGATTTTGCCAGCTAATGTAGCTCAAGATCATGCTCCAGAGGTTTTGAAGACTT
TGCAACAAATCAAAATGTTGGCTGATCCTGCTCCAAAAACAGTTTTGGGTTTGTCTAATGT
TTCTCAAAATTGCCAGAATAGACCTTTGATTAATAGAACTTTTTTGGCTATGGCAATGGCA
TGTGGATTGGATGCTGCTATTGCTGATGCTTGTGATGAAGCATTAATTGAGACTGCAGCT
ACCGCTGAAATTTTGTTGAATCAGACTGTTTATTGTGACTCTTTTGTTAAGATGTTTAAAA
CTAGATAActcgag

MLIIGERINGMFGDIKRAIQERDPAPVQEWARRQEEGGARALDLNVGPAVQDKVSAMEWLV
EVTQEVSNLTLCLDSTNIKAIEAGLKKCKNRAMINSTNAEREKVEKLFPLAVEHGAALIGLTM
NKTGIPKDSDTRLAFAMELVAAADEFGLPMEDLYIDPLILPANVAQDHAPEVLKTLQQIKML
ADPAPKTVLGLSNVSQNCQNRPLINRTFLAMAMACGLDAAIADACDEALIETAATAEILLNQT
VYCDSFVKMFKTR