THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

A study of ethylene production via the 2-oxoglutarate dependent pathway in *S. cerevisiae*

- insights from enzyme engineering, metabolic modeling and cultivation studies

NINA JOHANSSON

Systems & Synthetic Biology

Department of Chemical and Biological Engineering

CHALMERS UNIVERSITY OF TECHNOLOGY

Gothenburg, Sweden 2014

A study of ethylene production via the 2-oxoglutarate dependent pathway in *S. cerevisiae*
Insights from enzyme engineering, metabolic modeling and cultivation studies

Nina Johansson

© Nina Johansson, 2014

ISBN 978-91-7597-075-2

Doktorsavhandlingar vid Chalmers tekniska högskola,
Ny serie nr 3756
ISSN 0346-718X

Systems & Synthetic Biology
Department of Chemical and Biological engineering
Chalmers University of Technology
SE-412 96 Gothenburg
Sweden

Telephone: +46 (0)31 772 1000

Cover illustration by Karl Persson

Printed by Chalmers Reproservice
Gothenburg 2014

A study of ethylene production via the 2-oxoglutarate dependent pathway in *S. cerevisiae*
Insights from enzyme engineering, metabolic modeling and cultivation studies

Nina Johansson

Systems & Synthetic Biology
Department of Chemical and Biological engineering
Chalmers University of Technology, Sweden

Abstract

The detrimental effect of the petroleum industry on the environment combined with the threat of peak oil has driven the exploration for alternative strategies to produce traditional petrochemicals. Biotechnological production could be an alternative, using microorganisms to convert renewable feedstocks into desired products. A microbial based system for production of the traditional petrochemical ethylene has previously been developed through the expression of a bacterial version of the ethylene forming enzyme (EFE), which catalyzes the 2-oxoglutarate dependent ethylene pathway, in the yeast *Saccharomyces cerevisiae*.

This work aims at deepening the understanding of how the EFE functions and investigate the functionality of the *S. cerevisiae*-EFE cell factory for ethylene production. To this end metabolic modeling, metabolic engineering as well as several cultivation studies have been performed. Alongside this the enzyme has been characterized through structural prediction and enzyme engineering, which has reviled both a structural entity necessary for ethylene forming functionality as well as a number of specific amino acid residues coupled to ethylene formation.

Cultivation studies combined with metabolic engineering strategies have shown that balancing of arginine availability is important for optimal ethylene productivity. Further studies have also revealed that maintaining a high oxygenation level is a crucial cultivation factor for optimal ethylene productivity. This can be linked both to the reaction mechanism of the EFE, for which oxygen is a substrate, but also to an increased requirement of NADH re-oxidation when EFE is expressed. It was found that co-expression of heterologous oxidases could help relieve the redox stress and expression of the Aox1 of *Histoplasma capsulatum* was concluded to increase the ethylene yield with 28 %. To find further metabolic targets for increased ethylene productivity metabolic modeling was performed. The majority of the targets found were involved in supply of the EFE substrate 2-oxogltuarate, however none of the targets evaluated *in vivo* so far has given any increase in ethylene yields. Through this work important factors for optimal ethylene formation have been revealed, however it has also shown that more work is required before this system is a competitive alternative for ethylene production.

Keywords: Ethylene, *Saccharomyces cerevisiae*, ethylene forming enzyme, 2-oxoglutarate, production, cultivation, enzyme engineering, metabolic modeling, nitrogen metabolism, respiration rate

List of publications

This thesis is based on the following research papers, referred to as **Papers I-IV** in the text:

Paper I

Nina Johansson, Karl O Persson, Christer Larsson and Joakim Norbeck (2014)

Comparative sequence analysis and mutagenesis of Ethylene forming enzyme (EFE) 2-oxoglutarate/Fe(II)-dependent Dioxygenase Homologs

Accepted in BMC Biochemistry

Paper II

Nina Johansson, Paul Quehl, Joakim Norbeck and Christer Larsson (2013)

Identification of factors for improved ethylene production via the ethylene forming enzyme in chemostat cultures of *Saccharomyces cerevisiae*

Microbial Cell Factories 12:89

Paper III

Nina Johansson, Karl O Persson, Paul Quehl, Joakim Norbeck and Christer Larsson (2014)

Ethylene production in relation to nitrogen metabolism in *Saccharomyces cerevisiae*

FEMS Yeast Research, e-published ahead-of-print. DOI: 10.1111/1567-1364.12208

Paper IV

Nina Johansson, Karl O Persson, Gilles Vieira, Ludovic Cottret, Stéphanie Heux, Joakim Norbeck and Christer Larsson (2014)

In silico based metabolic engineering strategies for enhance ethylene productivity in *Saccharomyces cerevisiae* expressing the bacterial EFE

Manuscript

Contribution manuscript

Paper I:

Supervised experimental work, performed some of the experimental work, analyzed data and took part in writing the manuscript

Paper II:

Performed most of the experiments, supervised the remaining experimental work, analyzed data and wrote manuscript

Paper III:

Performed most of the experiments, supervised the remaining experimental work, analyzed data and wrote manuscript

Paper IV:

Performed many of the experiments, supervised the remaining experimental work, analyzed data and wrote manuscript

Preface

This dissertation serves as partial fulfillment of the requirements for a PhD degree at Chalmers University of Technology, Sweden. The PhD project was initiated as part of the EU-project BIOCORE and covers the characterization and evaluation of ethylene production via a metabolically engineered yeast strain. The work was carried out under the supervision of Professor Christer Larsson and Associate Professor Joakim Norbeck.

This research was mainly funded by grant agreement n°FP7-241566 of the European Community's Seventh Framework Programme (FP7/ 2007-2013). Personal funding has been received from the Nils Pihlblad Foundation, Adlerbertska Research Foundation and Ångpanneföreningens Research Foundation.

Nina Johansson
September 2014

TABLE OF CONTENTS

INTRODUCTION 1
ETHYLENE PRODUCTION – an overview 4
1.1 Traditional petrochemical production 5
1.2 Ethanol based production 5
1.3 The plant pathway 7
1.4 Microbial pathways 8
1.4.1 The EFE reactions and subsequent classifications 10
1.4.2 The EFE structure and sequence 12
1.4.3 The EFE reaction mechanism 16
LINKING THE PHYSIOLOGY OF S. CEREVISIAE TO ETHYLENE PRODUCTION 19
2.1 Saccharomyces cerevisiae 19
2.2 Carbon metabolism 20
2.2.1 2-oxoglutarate – mitochondrial and cytosolic pool 23
2.3 Respiration and/or fermentation 23
2.3.1 Regulation of the central carbon metabolism 24
2.4 Nitrogen metabolism 25
2.4.1 Selected amino acid metabolism 26
2.5 Preferred nitrogen source utilization 27
2.6 Growth in batch vs chemostat 29
2.7 Ethylene toxicity and transportation 30
2.8 Ethylene production in bioreactors 31
2.8.1 Ethylene sampling in different culture set-ups 31
2.9 Effect of nitrogen source on ethylene production 32
2.10 Influence of growth and respiration rate on ethylene formation 34
2.11 Some additional factors evaluated 36
METABOLIC IMPROVEMENTS OF THE S. CEREVISIAE-EFE CELL FACTORY 37
3.1 Metabolic engineering 37
3.1.1 Impact of rewiring of the nitrogen metabolism on ethylene formation 38
3.2 Metabolic modeling 40
3.2.1 S. cerevisiae models 41
3.3 Metabolic modeling & engineering of the S. cerevisiae-EFE cell factory 42

CONCLUSIONS 47
FUTURE DIRECTIONS 49
ACKNOWLEDGEMENT 51
REFERENCES 53

LIST OF FIGURES AND TABLES

Figure 1. Products derived from ethylene 4
Figure 2. Biological ethylene biosynthesis pathways 8
Figure 3. Schematic representation of the proposed mechanism of EFE 11
Figure 4. A predicted structure of the *P. syringae* EFE 13
Figure 5. ^{15}N-HSQC NMR-spectrum of EFE (unpublished data) 14
Figure 6. Ethylene production relative to wt of mutated versions of EFE 16
Figure 7. Effect of dissolved oxygen on ethylene productivity 17
Figure 8. Central carbon metabolism of *S. cerevisiae* 22
Figure 9. Selected parts of the nitrogen metabolism of *S. cerevisiae* 27
Figure 10. Arginine uptake in mixed nitrogen source cultivations 29
Figure 11. Production pattern in batch cultures depending on nitrogen source 33
Figure 12. Growth and product characteristics with increasing dilution rate 34
Figure 13. Arginine metabolism in *S. cerevisiae* 38
Figure 14. Effect of arginine on OD and ethylene production in a *Δarg4* strain 39
Figure 15. Metabolic engineering targets 43

Table 1. Properties of the *P. syringae* EFE 10
Table 2. Effect of nitrogen source on ethylene formation in chemostat cultivations 32
Table 3. Effect of respiration rate on ethylene formation 35
Table 4. Ethylene production in strains expressing heterologous oxidases 44
Table 5. FVA results of metabolic model iAZ900 extended with the complete EFE reaction 46

För dig

den vinner som är trägen, den förlorar som ger upp, drömmarna och vindarna hjälps åt

Lars Winnerbäck, Elden

INTRODUCTION

The petroleum industry is central in today's world, and products derived from it permeate most corners of our everyday lives; from fueling our cars to chemicals in hygienic products to building materials to derivatives found in food. However, the production, environmental and social sustainability of the petroleum industry is under questioning and a shift from an oil-based to a bio-based economy is increasingly asked for (European Commssion, 2012; OSTP, 2012).

One of the cornerstones for this shift is white biotechnology, i.e. the use of microorganism or enzymes for product manufacture, often with biomass envisioned as carbon source. The use of microorganisms for production purposes is not new and already in ancient times microorganisms were used within food processing, albeit unknowingly. In the early 20th century microbial fermentation began to be employed on an industrial scale for chemical production. Through the development of genetic engineering tools in the 70's it became possible to alter the inherit metabolism of the microorganism to increase product formation. These technologies also enabled production of heterologous compounds, i.e. genes from other origins are expressed in the microorganism making it possible to produce compounds not naturally formed by the host. Pushed on by the right to patent genetically modified organisms in 1980 (U.S. supreme court, 1980) the bio-chemical area has expanded immensely in the last decades and today biotechnological production of a vast variety of products is being explored, from jet fuel to pharmaceutical compounds (Hong and Nielsen, 2012).

Of all the organic compounds produced in the world, ethylene is claimed to be the one with the highest production volumes (McCoy et al., 2006), primarily due to large usage of its polymer products. In 2012 the global consumption of ethylene on a yearly basis reached 124 million tons and the demand keeps increasing (Koottungal, 2012; Mann et al., 2010; True, 2012). The traditional production is aside from being petroleum based also highly energy demanding and a major CO_2 producer. A biotechnical production method would hence be of great interest. Several different biological pathways to form ethylene do exist in nature as ethylene is a plant hormone as well as a virulence factor produced by several plant pathogenic microorganisms (van Loon et al., 2006; Weingart and Volksch, 1997; Yang and Hoffman, 1984). Throughout this study a single enzyme microbial pathway forming ethylene from 2-oxoglutrate has been employed.

A successful biotechnical production must combine the production pathway with a host organism which is suitable for industrial fermentation conditions and requirements, the choice therefor fell on the industrially well-established yeast *Saccharomyces cerevisiae*. Heterologous product formation however imposes several challenges. It requires not only that a possible route from metabolite to product is assembled, but a holistic understanding of the system must be in place to enable optimization of the production system.

The aim of this PhD was to study and characterize the biotechnical ethylene production system consisting of a microbial ethylene-forming-enzyme (EFE) expressed in *S. cerevisiae* and to both deepen our understanding of how the constituents of the systems functions and interact as well as get a grip on the functionality of the system as a biotechnical solution for ethylene formation. To meet these criteria the system has been studied at different levels. Large efforts have been made to find out more about the EFE itself. Full structural determination proved difficult, however through sequence analysis and structural comparison with related enzymes structurally important signatures for ethylene formation has been identified. A previously unknown ethylene producer was also identified (**Paper I**).

In **paper II** I studied several cultivation factors which affect the ethylene productivity, and identified some crucial factors to achieve optimal ethylene production. In this study it was also found that increased availability of the substrate/co-factor arginine had a detrimental effect on ethylene formation. In **Paper III** I investigated how this effect could be met and tried to understand the underlying mechanism for the effect and to identify a possible explanation.

I have also provided cultivation data used for optimizing a metabolic model of the system. Through this modeling work several metabolic targets were identified which were predicted to lead to increased ethylene production. A selection of these was subsequently tested *in vivo* in **Paper IV**. Further, as respiration rate had been identified as a possible limitation in a previous model, a strategy to overcome this was also evaluated in the same paper.

This thesis hence covers several different research areas; including metabolic engineering, yeast as a cell factory for a heterologous chemical formation, enzyme structure and function and the metabolism of *S. cerevisiae* during different cultivation setups. In the first part of this thesis I will give an overview of these areas and try to set my own research into perspective.

This part is divided into three chapters of which the first (**chapter 1**) summarizes how ethylene is produced today and could be produced in a microbial setting. It further gives a detailed description of the EFE and how it functions. **Chapter 2** links the production of ethylene via EFE to the metabolism of *S. cerevisiae* and elaborates on how the metabolism and physiological state of the yeast effects the ethylene formation. While **chapter 3** provides methods for identifying possible metabolic improvements of the combined yeast-enzyme ethylene factory as well as evaluates some of the identified targets. The second part of this thesis is comprised of the research articles which have come out of these studies.

CHAPTER 1

ETHYLENE PRODUCTION – an overview

The first account of ethylene (ethene) is usually ascribed the German physician J.J. Becher who is said to have described the gas in a book from the mid-17th century (Key, 1945; Livingstone, 1955). In the following century the chemical formula (C_2H_4) and structure - an alkene, i.e. the two carbons are connected by a double bond and each carries two hydrogens - of it was determined. The simple chemical formula combined with the double bond makes ethylene a good starter molecule to build on and ethylene has hence become a bulk and base chemical (Matar and Lewis, 2001a) (Fig. 1). In this chapter I will review the different production pathways of ethylene in use today and discuss the benefits and drawbacks of each of these. I will reason around the chosen production pathway and discuss the function and to some extent the structure of the ethylene-forming-enzyme (EFE) which has been employed.

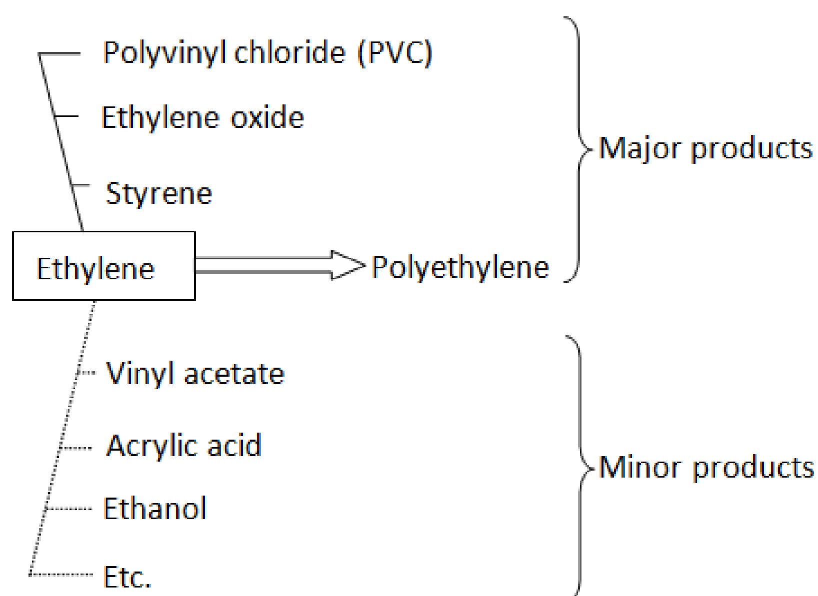


Figure 1. Products derived from ethylene

The double bond of ethylene is susceptible to a variety of derivatization reactions, e.g. polymerization, oxidation, halogenation and aromatic substitution, making ethylene a good building block chemical. The majority of ethylene is used for the production of differnet polymeric products

1.1 Traditional petrochemical production

Industrially the majority of ethylene is produced via pyrolysis (steam cracking) of naphtha, i.e. mixtures of hydrocarbons distilled from petroleum, though also other petroleum fractions and natural gas fractions are important feedstocks. In the cracking process the feedstock streams are preheated and mixed with steam, commonly at a 1:1 ratio of steam and hydrocarbon stream, then treated at temperatures of 750-850˚C for short time intervals (<0.5 sec). The ethylene yield in the process is dependent on the feedstock used. For naphtha the yield in the product stream is commonly around 35 mass-%, however if ethane is used as feedstock the cracking yield can reach 80-85 mass-%. (Matar and Lewis, 2001b). Even higher yields have been reported using catalytic cracking of ethane, through this process is not yet industrially very common due to issues with deactivation of the catalyst through e.g. coke deposition (Cavani et al., 2007). Ethane is becoming a feedstock of increasing importance as it can be extracted from natural gas, a resource for which the availability has increased greatly during the last decade due to hydraulic fracking (a.k.a fracking) of shale rock in foremost North America. In fracking natural gas entrapped deep underground is released using drilling, high pressure water, sand and chemical solutions (Holloway and Rudd, 2013). The increase in access of ethane has led to a sharp decrease in ethylene raw material price, however concerns have also been raised about the environmental effect of the technique, and studies have indicated that the fracking can lead to methane contamination of drinking water (Osborn et al., 2011) and increased seismic activity (Bame and Fehler, 1986).

What can be concluded is that traditional petrochemical based production of ethylene is a highly efficient and optimized process, although the feedstock extraction processes are linked to adverse environmental effects. Further the process is one of the most energy intense chemical processes in used today (Worrell et al., 2000).

1.2 Ethanol based production

As stated in the introduction above, many governments strive towards a more bio-based society and economy. In line with this a production process for ethylene has been developed in which ethanol is dehydrated catalytically to form ethylene. Bioethanol formation through fermentation of sugarcane or corn is a well-developed technology and ethanol is the dominating biofuel on the market at this point. However, as the feedstocks employed also function as or compete with food/feed, a shift towards non-competitive feedstocks, e.g. agricultural and forestry residues, is required for bioethanol and biochemicals in general to

reach their full potential (Sims et al., 2010; Tilman et al., 2009). Fermentation of lignocellulosic material (e.g. wood) encounters several difficulties such as high levels of inhibitory compounds and high dry matter concentrations (Koppram et al., 2014). While the starch and sugar based fermentation processes for ethanol in place today are efficient with final ethanol concentrations of 8-12 v-% and yields close to the maximal theoretical yield of 0.51g ethanol / g glucose (Basso et al., 2011; Galbe et al., 2007), the ethanol yields and final concentrations for the lignoscellulosic process are so far lower (Ask et al., 2012; Galbe et al., 2007; Gu et al., 2014). Extensive research is hence at the moment performed within the area of detoxification and fermentation efficiency.

The second step of the process is the catalytic dehydration of the formed ethanol. The catalytic mechanism of ethanol dehydration is a three step process, first the catalyst protonates the hydroxyl group which is disposed as water, the methyl group is subsequently deprotonated by the catalyst and finally the hydrocarbon rearranges into ethylene. During the past 15 years or so much effort has been made to optimize the catalyst composition to improve yields and operational costs. Traditionally γ-alumina was used as catalyst, however this gave low ethylene yields (80%) and required high temperatures (450˚C) (Fan et al., 2013). These days catalysts giving yields above 99% are available. The most promising of these are nanoscale HZSM-5 ($H_nAl_nSi_{96-n}O_{192}\cdot 16H_2O$, $0<n<27$) and $Ag_3PW_{12}O_{40}$ salt, both of which requires much lower temperatures than the traditional γ-alumina, 240˚C and 220˚C respectively (Bi et al., 2010; Gurgul et al., 2011). Despite being major improvements, the temperatures required are still high and hence so is the energy demand. Further, deactivation of the catalyst due to coke deposition, especially at higher moisture contents, is an issue which could lead to requirement of regeneration of the catalyst at narrow intervals (Aguayo et al., 2002; Zhang and Yu, 2013).

Interestingly, life cycle assessments of the above discussed ethylene production processes; cracking of naphtha, cracking of ethane and corn based bioethanol dehydration, concluded that the cumulative emissions of the different processes were similar and that the major part of the environmentally adverse effect stems from the production of the energy required by the processes. In the case of ethanol dehydration two of the most energy demanding steps are the distillation of ethanol from the fermentation broth and the catalytic dehydration step (Ghanta et al., 2013). This, hence, supports our work to develop a biological process which can convert biomass into ethylene in a one-step process. Several biological pathways exist in nature which could be employed in this direct conversion.

1.3 The plant pathway

In nature ethylene is most commonly known as a plant hormone being both sensed and produced by the plant. The biosynthetic pathway of ethylene in plants is well studied and two key enzymes have been identified; ACS (ACC synthase) and ACO (ACC oxidase). In summary ACS first converts the methionine derivative SAM (S-adenosyl methionine) into ACC (1-aminocyclopropane-1-carboxylic acid) and methylthioadenosin. The latter is recycled in the Yang cycle to reform SAM while ACC is further degraded by ACO into ethylene, CO_2 and HCN (Adams and Yang, 1979; Yang and Hoffman, 1984) (Fig. 2). Both ACS and ACO belong to strongly regulated multigene families, with the number of genes differing between plant species. The members of both the ACS and ACO families exhibit distinct temporal and spatial expression patterns and their activities and ethylene formation is affected by several factors such as the levels of other plant hormones and light. Further, several stress factors will also induce ethylene formation, including both abiotic (e.g. flooding and drought) and biotic ones (for reviews see Argueso et al., 2007; Lin et al., 2009).

The sensing of ethylene in plants is another complex story. The number of ethylene receptor isoforms present differs between plant species (e.g. *A. thaliana* have five versions while tomato has six) with overlapping but not redundant functions. The receptors are divided into two sub-families based on the sequence of the ethylene binding domain. They are membrane bound and commonly localizes to the ER, however alternative localizations have been determined for some isoforms. A copper cofactor is crucial for the binding of ethylene to the active site. In the inactive state the receptor forms a complex with the downstream repressor CTR1. Upon binding of ethylene the CTR1 will be released and inactivated and the signaling pathway de-repressed, with a diverse array of genes being the target for the regulation (for reviews see Binder, 2008; Wang et al., 2013; Zhao and Guo, 2011).

An attack from a plant pathogen will result in a sharp increase in ethylene production by the plant. Deficiency in the ethylene signaling results in a reduced resistance towards the infection of the plant (McManus, 2012). Interestingly, several plant pathogens also produce ethylene. In this case as a virulence factor and it has been found that some ethylene negative strains has significantly reduced infection rates (van Loon et al., 2006; Weingart et al., 2001). Ethylene hence has an ambiguous roll in infection development and the complete complexity of it has not yet been unraveled.

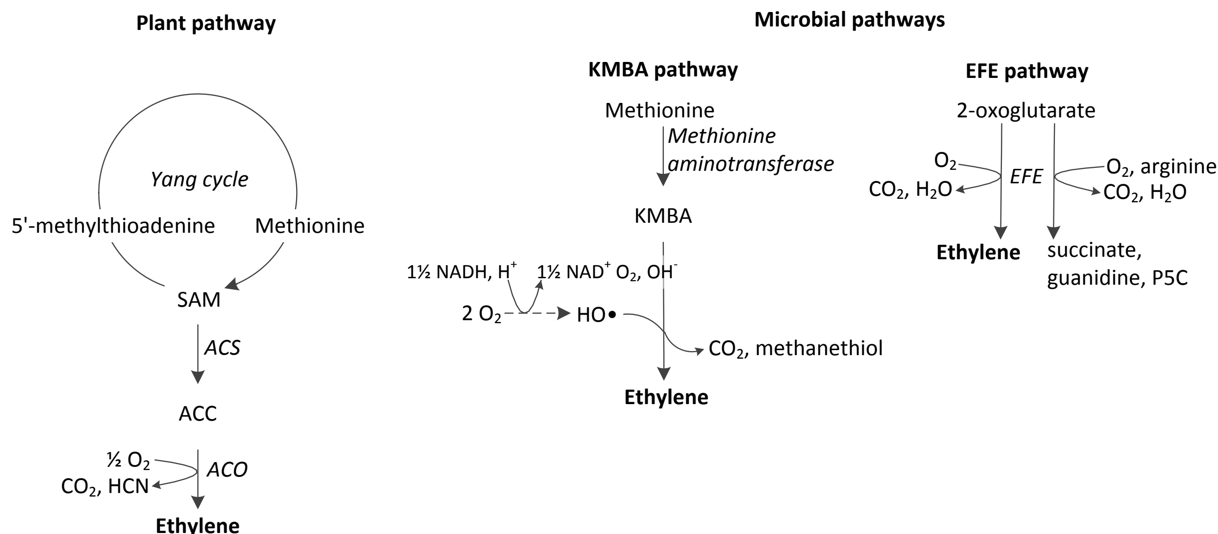


Figure 2. Biological ethylene biosynthesis pathways
The three known version. The plant version originates from methionine, likewise does one of the two known microbial pathways. The third version is the 2-oxoglutrate dependent pathway, found in some microorganism and the one utilized in this study. SAM; S-adenosyl methionine, ACC; 1-aminocyclopropane-1-carboxylic acid, ACS; ACC synthase, ACO; ACC oxidase, KMBA, 2-keto-4-methylthiobutyric acid, P5C; 1-Pyrroline-5-carboxylic acid, EFE; ethylene forming enzyme

1.4 Microbial pathways

That some microorganisms produce ethylene has been known since the mid 1900's when it was determined that the gas was formed by both the green fruit mold *Penicillum digitatum* (Biale, 1940; Miller et al., 1940; Young et al., 1951) and by the human pathogen *Blastomyces dermatitidis* (Nickerson, 1948). Since then numerous microbial ethylene producers have been discovered, from bacterial as well as fungal origins. Two pathways for ethylene production have been identified within these microorganisms; the L-methionine-dependent KMBA-pathway and the 2-oxoglutarate-dependent EFE-pathway. In the former L-methionine is deaminated into 2-keto-4-methylthiobutyric acid (KMBA) which through a complex reaction and formation of hydroxyl radicals is oxidized to ethylene. The latter pathway is catalyzed by a single enzyme, the ethylene forming enzyme (EFE) for which 2-oxoglurtarate is a key substrate (Fukuda et al., 1993; Ogawa et al., 1990) (Fig. 2).

Investigation of 757 strains of bacteria found that 30 % of these produced ethylene. A very vast majority of these (225) were determined to be forming ethylene via the KMBA pathway while only one was found to be utilizing the EFE pathway – a *Pseudomonas syringae* strain. When comparing the ethylene production rates of the 226 ethylene forming strains it was

concluded that the *P. syringae* strain exhibited the highest rate of all, indicating that the EFE pathway is the most efficient (Nagahama et al., 1992).

Several different pathovars of *Pseudomonas syringae* have been evaluated for their ethylene production efficiency via the EFE-pathway. The pathovar *phaseolicola* was found to be about twice as productive as the *glycinea, sesame* and *cannabina* ones, while the pathovar *pisi* produces considerably less and several others seems to have only minute production or even lacking production and the EFE gene completely (Sato et al., 1987; Sato et al., 1997; Weingart and Volksch, 1997; Weingart et al., 1999). The EFE mainly used in this study is of the *Pseudomas syringae* pathovar *phaseolicola*, hence one of the top ethylene producing strains found. Aside from this two fungal EFEs from *Penicillium* species (*P. digitatum* and *P. chrysogenum*) were also studied in **paper I**. It was found that when expressed through an integrative strategy in *S. cerevisiae* the EFE of *P. chrysogenum* is non-active (no ethylene formation seen). Further a new potential ethylene producer; *Myxococcus stipitatus*, was identified by performing a BLASTp search with the *P. syringae* EFE as query and looking for certain conserved motifs. The *M. stipitatus* protein was found to have trace production of ethylene, and as far as we know this is the first non-plant-pathogenic microbial ethylene producer identified (**paper I**).

EFEs of mainly *Pseudomonas syringae* origin has been heterologously expressed in a multitude of host organism including the prokaryotic; *Escherichia coli* (Ishihara et al., 1995), *Pesudomonas putida* (Ishihara et al., 1996; Wang et al., 2010), *Synechococcus* (Sakai et al., 1997; Takahama et al., 2003), *Synechocytis 6803* (Ungerer et al., 2012) and the eukaryotic; *Saccharomyces cerevisiae* (Pirkov et al., 2008), *Trichoderma viride* (Tao et al., 2008) and *Trichoderma reesei* (Chen et al., 2010) as well as within the plant *Nicotiana tabacum* (Araki et al., 2001). Overexpression of the EFE within the native producers has also been evaluated (Ishihara et al., 1996). A compilation of the ethylene production rates achieved so far reviled that the *S. cerevisiae*-EFE system is the second most efficient. The rates achieved with the *S. cerevisiae* have only been surpassed by multicopy integration in combination with vector expression of EFE in *P. putida* and the production rates with *S. cerevisiae* are almost twice as high as those reached within *E coli* (Eckert et al., 2014). The inherent suitability of *S. cerevisiae* in an industrial setting will be discussed in **chapter 2**.

Comparison of the plant pathway to the EFE dependent microbial pathway through metabolic modeling concluded that in theory the two pathways should produce ethylene approximately equally effective when expressed in *S. cerevisiae* (Larsson et al., 2011). However, actual expression of the plant pathway in *S. cerevisiae* resulted in production levels significantly lower compared with the EFE pathway in the same host (Lu et al., 1999; Pirkov et al., 2008). One explanation for this lower production could be the side product HCN formed in the plant pathway which is highly toxic. The presence of HCN could further present a serious issue in industrial settings. As the alternative microbial pathway, the KMBA pathway, includes a non-enzymatic degradation step dependent on hydroxyl radicals, which are also toxic to the cell, the EFE pathway seems to be the most suitable of the biological options known today.

Table 1. Properties of the *P. syringae* EFE

Property		Reference
Molecular mass	42 kDa	(Nagahama et al., 1991b)
pI	5.9	(Nagahama et al., 1991b)
optimal pH for stability	7.0-7.5 [1]	(Nagahama et al., 1991b)
optimal temp. for catalysis	20-25˚C [1]	(Nagahama et al., 1991b)
	25˚C [2]	(Ishihara et al., 1995)
maximum temp for stability	30˚C [2,1]	(Ishihara et al., 1995; Nagahama et al., 1991b)
T_m* (in hepes with no substrates)	40˚C	**This work (unpublished)**
T_m with arginine	42.5˚C	**This work (unpublished)**
T_m with 2-oxoglutarate	45 ˚C	**This work (unpublished)**
K_m Fe^{2+}	$5.9*10^{-5}$ M	(Nagahama et al., 1991b)
K_m L-arginine	$1.8*10^{-5}$ M	(Nagahama et al., 1991b)
K_m 2-oxoglutarate	$1.9*10^{-5}$ M	(Nagahama et al., 1991b)

* Based on differential scanning fluorimetry. Increased T_m correlates to a stabilizing effect of the additive on the enzyme indicating binding of the additive to the enzyme.
[1] *in vitro*
[2] *in vivo* (*E. coli*)
[3] *in vivo* (*S. cerevisiae*)

1.4.1 The EFE reactions and subsequent classifications

The EFE has been thoroughly characterized (Table 1). It was early concluded that it requires the presence of not only 2-oxoglutarate but also Fe(II), oxygen and arginine for the formation of ethylene. Moreover, it was determined that the addition of chelating reagents resulted in loss of ethylene formation from which it was proposed that the Fe(II) has a coordinating function in the enzyme. (Nagahama et al., 1991a; Nagahama et al., 1991b). In one of the

studies Nagahama et al. (1991a) propose a reaction mechanism of the EFE, in which the active site is an Fe(II) complex. When coordinated with the 2-oxoglutarate and arginine a so called Shiff's base ($R^1R^2C{=}NR^3$) intermediate is formed which upon the addition of oxygen decomposes to one ethylene and two CO_2. In a later study a second reaction was added to the scheme as it was found that succinate was also formed by the enzyme at a ratio of 1 succinate per 2 ethylene molecules. Further, arginine was consumed almost equally fast as the succinate was formed, from this it was concluded that in the succinate reaction arginine is a substrate, while in the ethylene reaction it is merely a co-factor. Hence, a dual circuit mechanism of the EFE was proposed, with the ethylene forming reaction occurring 2 times for each non-ethylene forming reaction, (Fig. 3).

The two reactions are chemically summarized as (Fukuda et al., 1992);

1) 2-oxoglutarate + O_2 → ethylene + 3 CO_2 + H_2O
2) 2-oxoglutarate + O_2 + arginine → succinate + 1-Pyrroline-5-carboxylic
+ acid guanidine + CO_2 + H_2O

With the average reaction of the enzyme being:

3) 3 2-oxoglutarate + 3 O_2 + arginine → 2 ethylene + succinate + guanidine +
1-Pyrroline-5-carboxylic acid + 7 CO_2 + 3 H_2O

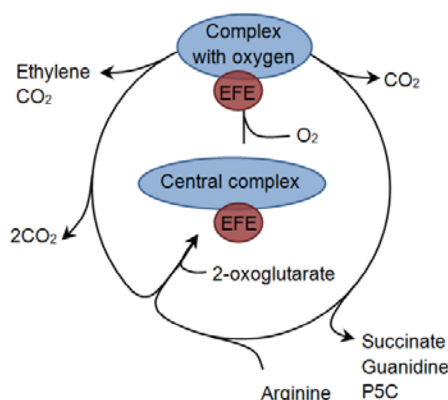


Figure 3. Schematic representation of the proposed mechanism of EFE
A dual mechanism in which the decomposition of the oxygen complex determines which product pathway of the two will be occurring.

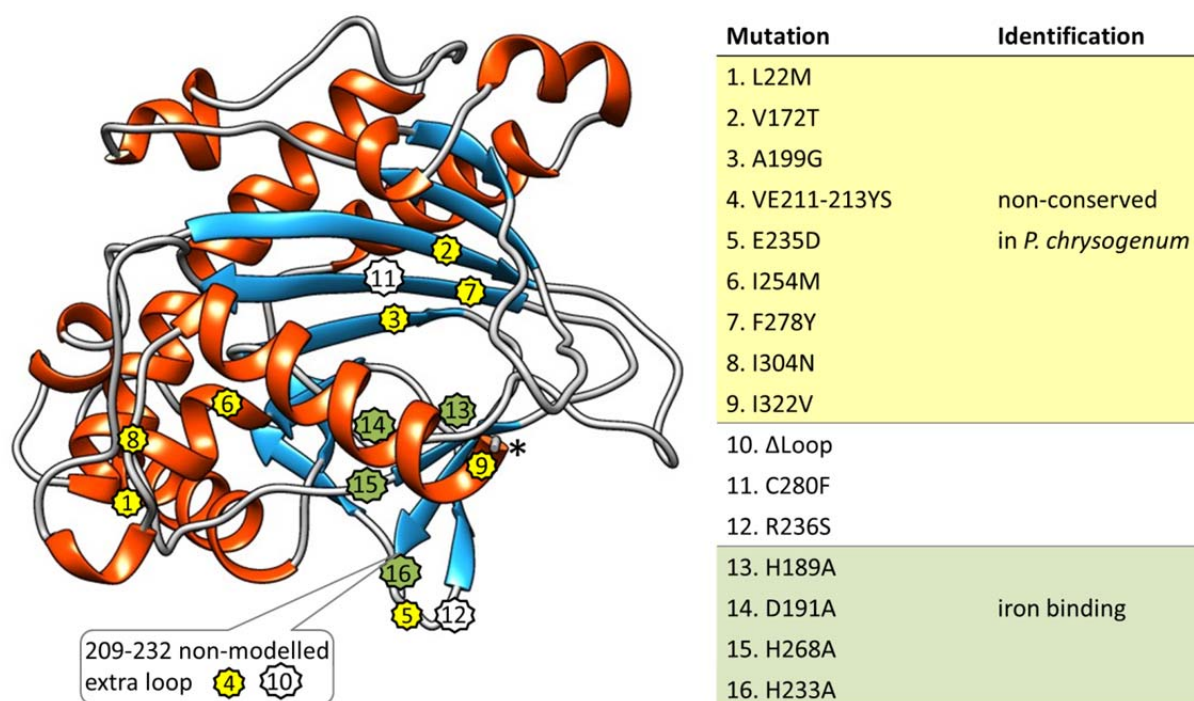
The two sub-reactions of the EFE classifies the enzyme within two different enzyme families: for reaction 1) the 2-oxoglutarate/Fe(II) dependent dioxygenases (Brenda 1.13.12.19 – one substrate oxogenase) and for reaction 2) the 2-oxoglutarate/L-arginine monooxygenase/decarboxylase (succinate-forming) family (Brenda EC 1.14.11.34 – two substrate oxogenase). The basis for both of these reactions is the central EFE complex with 2-oxoglutarate, O_2 and Fe(II) which puts the enzyme as a whole within the large and biologically diverse Fe(II)/2-oxoglutarate dependent oxygenase super family.

1.4.2 The EFE structure and sequence

The structure of the EFE is unknown and despite serious efforts during this work to structurally determine the *P. syringae* EFE it has not been possible to resolve the structure, neither through crystallization nor through NMR experiments, due to issues with stability as well as crystal formation. Instead a structure prediction through sequence homology with other structurally determined enzymes was made for the EFE sequences of *Pseudomonas syringae, Penicillim digitatum* and *Penicillium chrysogenum* using the program Phyre2 (Baker and Sali, 2001; Kelley and Sternberg, 2009). This predicted that the EFEs have good structural similarities with a Leucoanthocyanidin dioxygenase (LDOX) from *Arabidopsos thaliana* (**paper I**). From this model it was observed that the EFEs should contain the jelly roll structure which is unifying for the Fe(II)/2-oxoglutarate dependent oxygenases (Hausinger, 2004) (Fig. 4). This barrel-like structure is composed of pairs of antiparallel β-sheets wrapping to form the core of the enzyme (Hausinger, 2004; Roach et al., 1995). The jelly roll structure is stabilized by surrounding structures, in the case of EFE predicted to mainly be α-helices. The presence of α-helices was further supported by a ^{15}N-HSQC NMR-spectrum of the *P. syringae* EFE achieved within this work which exhibit a quite narrow peak dispersion typically indicative of α-helices (Fig. 5) (**unpublished data**).

Within the hydrophobic cavity of the jelly roll the coordinating iron of the enzyme complex is contained. The ion is generally connected to the enzyme peptide by weak interactions with the side chains of three amino acids. These residues typically follow the pattern His-X_1-Asp/Glu-X_2-His, X being a strech of spacing residues varying from enzyme to enzyme within the family (Hausinger, 2004). Histidine resiudes, through their N-atoms, are hence central for the coordination of the Fe-ion. Based on mutation experiments of 10 different histidine residues within the *P. syringae* EFE, H189 and H233 were proposed to be the ligands to the Fe ion as mutation of these led to complete loss of ethylene activity (Nagahama et al., 1998). However,

based on the sequence and structure analysis of the above three EFEs and comparison with related enzymes, it was peoposed that the ligands are likely rather H189 and H268, together with D191 (Hausinger, 2004) (**paper I**) (Fig. 4). This is supported by the fact that mutation of H189, H268 and D191 all respectively led to lost ethylene formation ability (**paper I**) (Fig. 6), whereas the H233A remained active. Plotting the H233 residue onto the predicted structure of EFE, it is further found that it is located away from the center of the enzyme (Fig. 4). Making it even less likely that it is involved in the Fe coordination.



Mutation	Identification
1. L22M	
2. V172T	
3. A199G	
4. VE211-213YS	non-conserved
5. E235D	in *P. chrysogenum*
6. I254M	
7. F278Y	
8. I304N	
9. I322V	
10. ΔLoop	
11. C280F	
12. R236S	
13. H189A	
14. D191A	iron binding
15. H268A	
16. H233A	

Figure 4. A predicted structure of the *P. syringae* EFE
as generated by the Phyre2 server. Plotted onto the structure are the sites of the mutations introduced to the enzyme. The table indicates which mutation each number correspond to. Yellow numbers show residues correlating with ethylene formation and green numbers to residues believed to be involve in the binding of the iron.
*amino acids 323-350 at C-terminal not in model

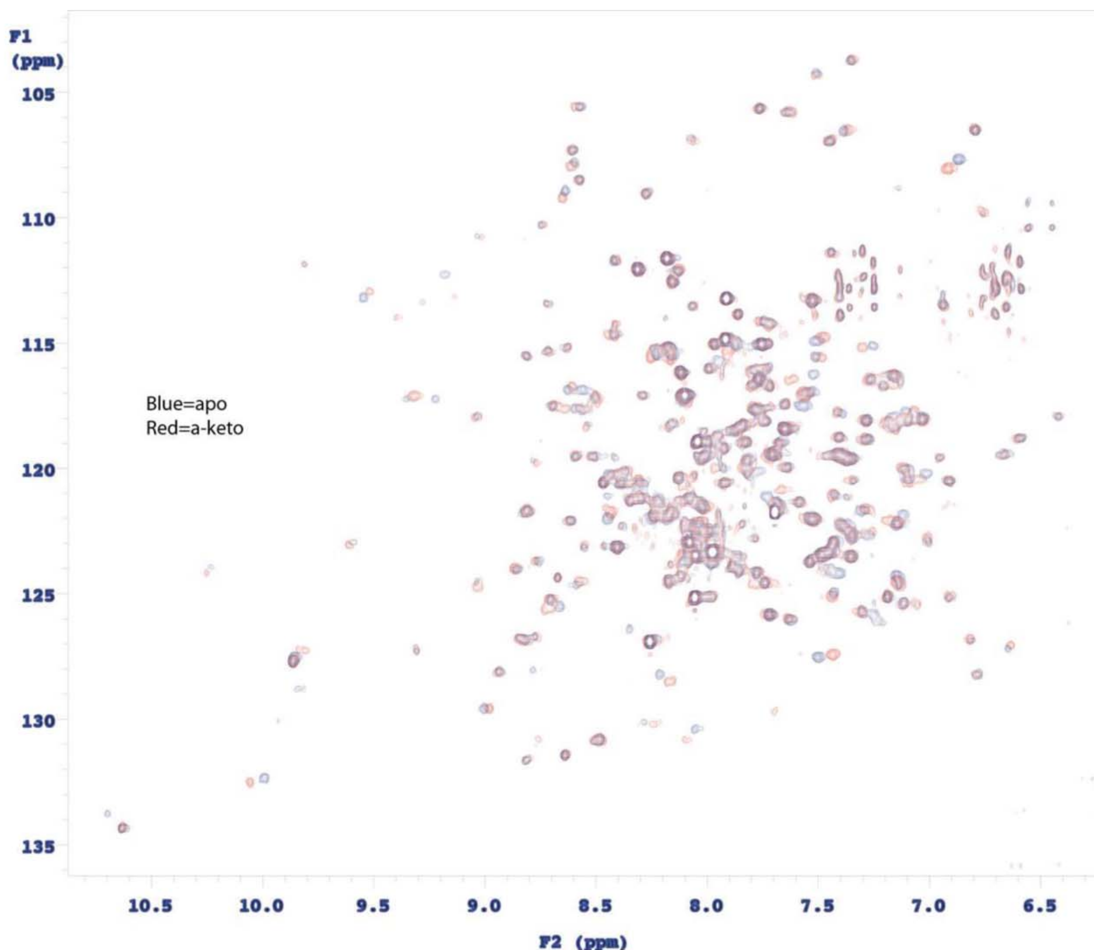


Figure 5. ^{15}N-HSQC NMR-spectrum of EFE (unpublished data)
The spectrum shows nitrogen-protons which are covalently bound, i.e. all amino acids except proline should give rise to a least one signal. Certain side chain will also give rise to a signal (i.e. Trp, Asn and Gln). However, due to the size of the enzyme some signals are too weak to register.
Blue spectrum; pure enzyme, red spectrum; enzyme with 2-oxoglutarate added, purple signifies overlaps between the two spectra.

Comparing the sequences of the two ethylene producing EFEs of *P. syringae* and *P. digitatum* with the sequence of the non-producing *P. chrysogenum* 10 residues could be identified which were conserved in the two functional, but not conserved in the non-functional (**paper I**). Several of these are of extra interest as they are found in crucial parts of the sequence. V172 and F278 (nomenclature based on the *P. syringae* sequence) are within suggested 2-oxoglutarate binding domains (when comparing to those found in the LDOX of *A. thaliana*) (Wilmouth et al., 2002), while A199, although not in any suggested binding domain, clusters structurally close to the two previous among the β-sheets making up one side of the active site (Fig. 4). Exchanging the residues of the two former ones with the ones found in the non-functional *P. chrysogenum* sequence had little effect on the enzymes ethylene productivity,

while altering the A199 to the glycine found in the *P. chrysogenum* EFE interestingly led to a substantial decrease in ethylene formation (Fig. 6), indicating that the A199 is important in assuring that the substrates bind effectively to the active site.

Inspection of the predicted structure further revealed a non-structured and non-modelled loop between residues 210 and 232 (Fig. 4). This loop was found to be required for ehylene formation (Fig. 6). Three non-conserved residues were further found in or close to the loop; V212, E213 and E235. A double mutant of the two neighbors 212 and 213 showed reduced capability for ethylene formation, again indicating that this characteristic loop is crucial for the function of the EFE.

The mutation which showed the largest effect out of the 10 was the I304N, for which ethylene formation was completely abolished (Fig. 6). This non-conservative mutation is situated just before the C-terminal α-helix and may hence affect the positioning of this, thereby possibly affecting the form of the active site and the ability to bind the coordinating iron (Fig. 4). Mutation of the final three of the 10 residues; L22, I254 and 1322 did not affect the ethylene formation (Figs. 4 and 6). Further, in an attempt to restore ethylene formation to the *P. chrysogenum* EFE, the 10 identified residues were altered into the amino acid found the *P. syringae* EFE. This was however not enough to reestablish ethylene formation to the enzyme.

Apart from the amino acids above, it was found that residue 280, which is close to one of the suggested 2-oxoglutarate binding motifs, is a cysteine in the *P. syringae* EFE whereas being a more bulky and hydrophobic phenylalanine in both the *Penicillium* versions of the enzyme. Exchanging the cysteine for a phenylalanine severely reduced the productivity of the *Pseudomonas* EFE. Further, comparing the sequence of the top ethylene producing *Pseudomonas* pathovar; phaseolicola, to the sequences of the pathovars cannabina, sesamii and glycinea it was found that they only differ at one position; R236 in phaseolicola is replaced with an S in the three other pathovars. This mutation also conferred a large decrease in ethylene production when introduced to the phaseolicola version, supporting the importance of this residue (Figs. 4 and 6).

From these tests it can conclude that mutations which seemingly effect the coordinating iron have the largest effect on the ethylene formation activity of the EFE, while mutations within substrate binding domains can effect, but commonly to a lesser extent.

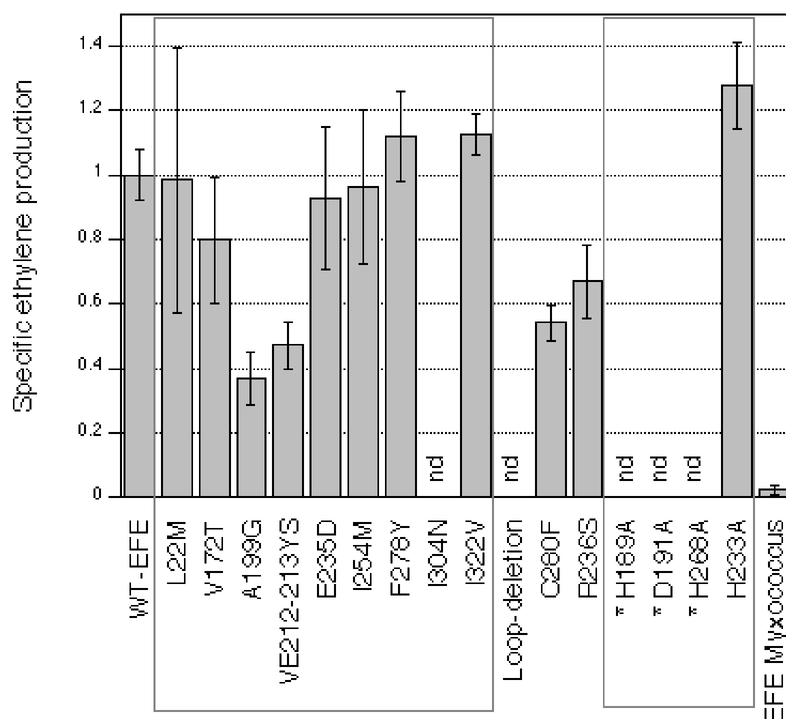


Figure 6. Ethylene production relative to wt of mutated versions of EFE
Large rectangle indicates the amino acids identified to correlate with ethylene formation, the small rectangle those believed to be involved in binding of the coordinating iron. Production of the wild type version was set to 1, standard deviations of at least three separate cultures.

1.4.3 The EFE reaction mechanism

From the NMR-spectra it could be concluded that when 2-oxoglutarate was added to the sample a shift in position of approximately 15 residues occurred (difference between red and blue marking in figure 5). This is typical for the binding of a small molecule such as 2-oxoglutarate. Within the oxogynase family 2-oxoglutarate is known to bind to the active site in two modes; In-line and Off-line, depending on how the C-1 carboxyl and C-2 keto groups of 2-oxoglutrate chelates the Fe-ion. Which binding mode that will occur is governed in part by interaction of the 2-oxoglutarate C-5 carboxylate with arginine and lysine residues and in part by C-1 carboxylate-Arg interactions (Hausinger, 2004). From the NMR spectrum it can, however, not be determined which of the two modes that is the functional for EFE. Further, the flexibility of the catalytic reaction that these two modes open up for could perhaps be part of the explanation for the dual-circuit mechanism proposed for EFE. Fukuda et al. (1992) hypothesized that the stretching of the bound 2-oxoglutarate molecule would favor the ethylene forming reaction of the EFE. Speculating freely, this stretching could perhaps be dependent on the 2-oxoglutarate binding mode

In the ethylene forming reaction arginine functions merely as a co-factor, while in the non-ethylene forming reaction it is a co-substrate. The reaction mechanism proposed by Fukuda et al. (1992) does not conform to any of the reaction mechanisms described for other enzymes within the family and it is hence questionable if this proposed mechanism is actually true. One major difference in the reaction pattern suggested for EFE compared to other enzymes within the family is how arginine is bound in the active site. Commonly for the enzymes in the family the co-substrate (in our case arginine) does not bind directly to the metal ion, but rather coordinates itself within the active site so as to be close to the 2-oxoglutarate-metal reactive center (Hausinger, 2004), unlike what is proposed for the EFE where the arginine is suggested to coordinate directly to the enzyme (Fukuda et al., 1992).

The final substrate to bind to the reactive site is oxygen which forms a reactive peroxo-structure in the EFE-complex. According to Fukuda et al. (1992) this decomposes irreversibly towards one of the two possible reaction pathways. The reactiveness of the oxygen intermediate combined with the flexibility in the 2-oxoglutarate binding gives that steering of the reaction towards the desired reaction likely is hard. Moreover, reactive oxygen forms are damaging for both the cell as a whole and for the enzyme itself. It has also been found that if the coordinating ion is oxidized before the binding of the substrates it can result in an inactive Fe(III) form (de Jong and Kemp, 1984). This could hence mean that a part of the EFE pool present is inactive. It can therefore be tempting to limit the oxygen provision to minimize detrimental binding of oxygen to the active site, however metabolic model results (Larsson et al., 2011) as well as actual experiments showed that a good provision of oxygen *in vivo* was required for optimal ethylene formation and no negative effect was seen even when relatively high oxygenation levels were applied (**paper II**) (Fig. 7).

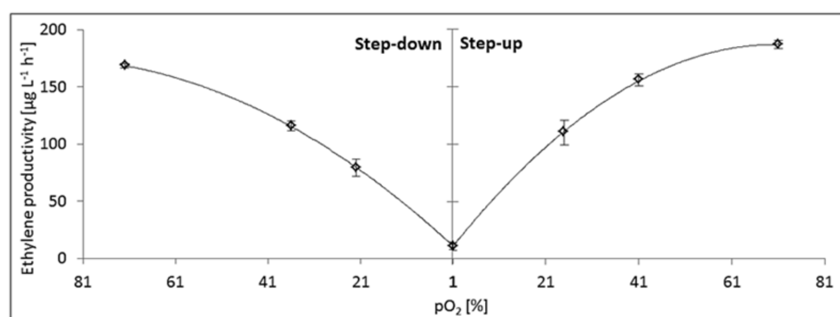


Figure 7. Effect of dissolved oxygen on ethylene productivity
as the oxygenation level is step-wise decreased followed by a step-wise increase.
100 % pO_2 is equal to the amount dissolved oxygen in air saturated water at 30˚C.

Taking all of the above in to account EFE seems the most promising of the three biological pathways, however even though we are learning more about the structure and function of the enzyme it still presents us with several challenges, including determining its true structure and reaction mechanism. Further its stability seems low and strategies to meet this, i.e. altering the source of the enzyme to one phylogenetically closer to *S. cerevisiae* or co-expressing the *P. syringae* chaperons GroEL and GroES (**unpublished data**), has rather decreased productivity.

CHAPTER 2

LINKING THE PHYSIOLOGY OF S. CEREVISIAE TO ETHYLENE PRODUCTION

Already in the ancient societies of Mesopotamia (McGovern et al., 1996) and pre-historic China (McGovern et al., 2004) humans seem to have been exploiting yeast (albeit unknowingly) to produce edible and drinkable products. In these early applications it was the ability to ferment sugar into alcohol and carbon dioxide which were sought after. Through work by scientist such as Anton van Leeuwenhoek, Robert Hooke, Lazzaro Spallanzani and Louis Pasteur it was established that the process was due to a small living entity – a microorganism – later classified as a yeast which was given the name *Saccharomyces cerevisiae*. Its metabolism and growth profiles came to be extensively studied and characterized.

In the last centuries a new area of utilization has arisen for *S. cerevisiae*, namely the industrial biotech area where it is used as host for chemical production of both natively and non-natively formed substances. This chapter will reason around why *S. cerevisiae* is a suitable host for chemical production. It will give an overview of its central metabolism and review its physiology in different growth conditions. I will further connect the metabolism and physiology to ethylene formation and discuss important findings we made regarding optimal growth conditions for ethylene production.

2.1 *Saccharomyces cerevisiae*

Through the years the yeast *S. cerevisiae* has emerged as one of the central microorganisms within the microbiology and biotechnology areas. Its long historical usage has given it some advantages over other organisms available, e.g. many metabolic engineering tools are available for it, its metabolism is one of the most studied, it was one of the first genomes to be sequenced (Goffeau et al., 1996) and several of the so called omics-techniques (e.g. genomics, metabolomics, fluxomics) are well developed for it. Thus, with time it has become one of our model organisms. *S. cerevisiae* moreover has some advantageous properties in industrial settings e.g. short generation times, ability to grow in both minimal and complex media, aerobic as well as anaerobic growth, growth at a relatively wide pH range and a GRAS status (generally regarded as safe) making it legally easier to handle and implement. *S. cerevisiae* is

hence both a scientific and an industrial work horse, supporting its use as the platform for the development of an ethylene cell factory.

A multitude of *S. cerevisiae* strains have been employed scientifically, typically dependent on specific research topic and research group traditions. Different strains can have important variations genetically and phenotypically, e.g. differences in protein expression (Rogowska-Wrzesinska et al., 2001) lipid metabolism (Daum et al., 1999) and missing genes (Daran-Lapujade et al., 2003) have been concluded during systematic analyses. An interlaboratory study with the aim of finding a common reference strain taking several aspects into account including growth rate, possible nitrogen and carbon sources, aerobic growth and respiration rate, genetic stability, sporulation efficiency etc. found that the strain family CEN.PK (which was originally developed specifically to meet the requirements of several research areas in an interdisciplinary project and which is based on two laboratory strains) represented a good compromise between the properties asked for and was subsequently chosen as a platform for cell-factory research (van Dijken et al., 2000). This is also the strain family employed throughout this work.

2.2 Carbon metabolism

Glucose is the preferred carbon source for *S. cerevisiae*, though several different carbon compounds, including other sugars as well as C2 and C3 compounds, can support growth of it. Some of these compounds, such as galactose, fructose, mannose, maltose and sucrose, are fermentable like glucose, whereas ethanol for example is non-fermentable by *S. cerevisiae* and requires aerobic conditions (Barnett, 1997). In previous chapter I discussed using biomass as the raw material. The main carbons source in this is glucose from the cellulose, however biomass also contains the sugar xylose. It has been shown that some *S. cerevisiae* strains are capable of metabolizing xylose at a low rate to support slow growth (Attfield and Bell, 2006; Wenger et al., 2010), however most xylose using strains have been developed by expressing heterologous pathways. Scientifically growth on glucose is the most studied for *S. cereviaise*.

The term metabolism is defined by the Oxford dictionary as "*The chemical processes that occur within a living organism in order to maintain life*" (Oxford Dictionaries, 2014) and is usually divided into catabolic (decomposing) and anabolic (synthesizing) reactions. One of the backbones of the metabolism of *S. cerevisiae* is the central carbon metabolism, through which the supplied (or available) carbon source is ultimately converted into biomass precursors and ATP (Noor et al., 2010). The system is composed of several metabolic

pathways whose activities are dependent on environmental factors such as presence of oxygen as well as on the type and sometimes amount of C-source present. As glucose is the preferred carbon source this is also the most commonly used. The metabolism of glucose starts with the uptake of the molecule from the surrounding environment. This occurs through facilitated diffusion via one of the many hexokinases present in the cell. The affinity for glucose of these transporters spans from low to high (K_m 100 mM to K_m 1 mM) giving that glucose can be taken up even at small externals concentrations (Reifenberger et al., 1997). The internalized glucose is then metabolized into pyruvate in what is known as the glycolysis, a pathway which has been found to be metabolically highly optimized (Bar-Even et al., 2012). In the glycolysis two ATPs are formed through so called substrate phosphorylation where a phosphate group is transferred from a pathway intermediate to ADP to form ATP. The pathway further supplies two reducing equivalents in the form of NADH.

Under anaerobic conditions the formed pyruvate will be converted via pyruvate decarboxylase into acetaldehyde which is subsequently reduced into ethanol by alcohol dehydrogenase using NADH as electron donor, thereby giving a redox neutral process for glucose degradation (Fig. 8). Under this conditions all the cells ATP is hence derived through the glycolysis. Under aerobic conditions the pyruvate is instead transported into the mitochondria where pyruvate dehydrogenase converts it into acetyl-CoA, which in turn combines with oxaloacetate to form the first step of the tricarboxylic acid cycle (TCA cycle). Pyruvate hence makes up the branch point between fermentative (anaerobic) and respirative (aerobic) growth. Further, as the TCA cycle will be partially drained on some intermediates as these are used in the formation of certain amino acids (Jones and Fink, 1982) pyruvate can also be used to replenish the oxaloacetate pool through the pyruvate carboxylase (Fraenkel, 1982; Pronk et al., 1996) (Fig. 8). This might be extra important when the EFE is introduced as it might cause an extra drainage of the intermediate 2-oxoglutarate as this is a substrate of the enzyme.

In the TCA cycle the acetyl group of the acetyl-CoA is oxidized through conversions via a number of acid intermediates. In the process several reducing equivalents (NADH and $FADH_2$) are formed. These equivalents will function as electron donors in the electron transport chain in which the donated electrons are passed through a series of carrier molecules of increasing electronegative potential finally reducing molecular oxygen to water. During the process of electron transfer, the release of Gibbs free energy is utilized to pump protons across the inner mitochondrial membrane, thus forming an electrochemical gradient. The proton motive force of this gradient is utilized by the ATPase to form ATP. The process is

known as oxidative phosphorylation as the electron donors are oxidized upon the transmission of the electrons. As respirative growth generates ATP in both the glycolysis and the electron transport chain it hence provides more ATPs/glucose than does fermentative metabolism. Also, as the glucose is fully converted to biomass and CO_2 under aerobic conditions, in contrast to under anaerobic were parts of the carbon will go to ethanol formation, the biomass yield is higher; usually 0.5 g biomass / g glucose under aerobic conditions compared to only 0.1 biomass / g glucose during anaerobic growth (Fiechter et al., 1981; Käppeli, 1986; Rieger et al., 1983a)

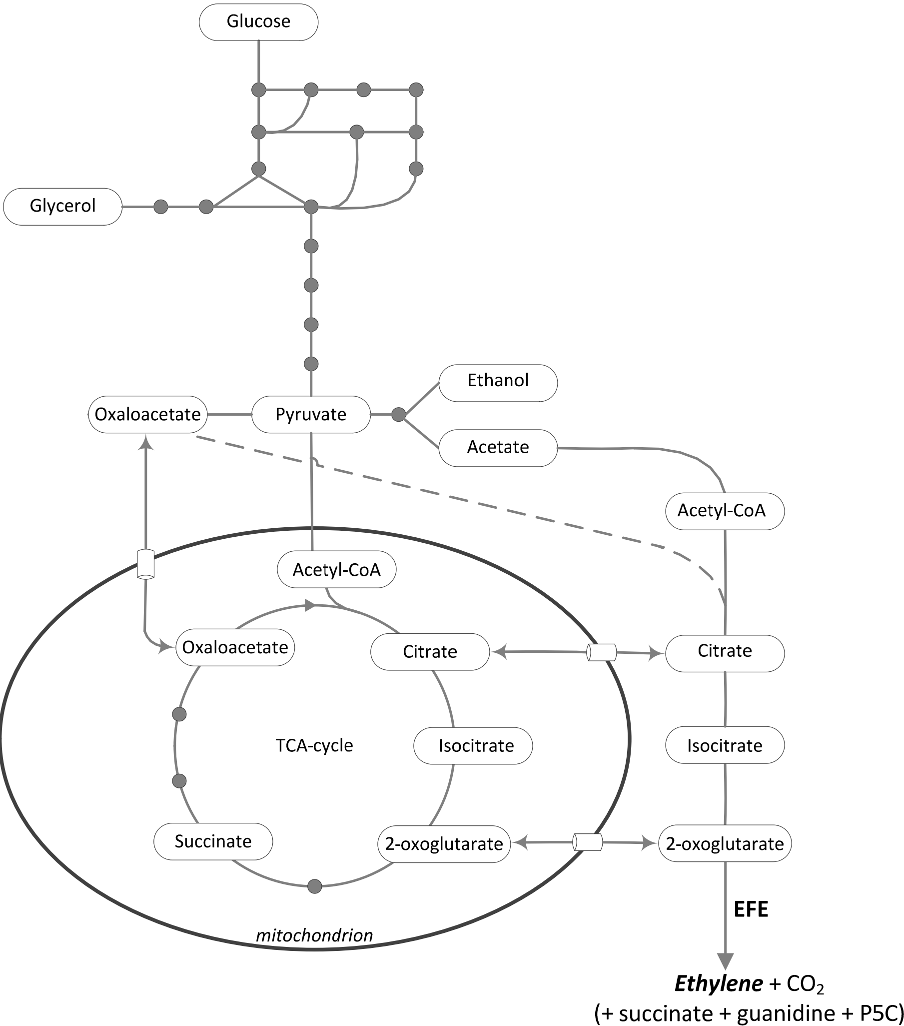


Figure 8. Central carbon metabolism of *S. cerevisiae*
Linked to ethylene formation via EFE. Each dot indicates a new metabolite in the pathway.
Metabolites of specific interest are written out.
Cytosolic expression of EFE has been employed throughout this study.

2.2.1 2-oxoglutarate – mitochondrial and cytosolic pool

The EFE substrate 2-oxoglutarate is an important intermediate of the TCA cycle and during respirative growth there will hence be a mitochondrial pool of the compound. As pyruvate makes up the branch point between respirative and ferementaive growth, one could draw the conclusion that there would be no flux through the TCA cycle during fermentative growth and hence that the formation of 2-oxoglutarate would be limited, however 2-oxoglutarate is an important metabolic precursor for formation of amino acids of the glutamate family and is thus always required by the cell for proliferation (Cooper, 1982). Flux analysis of anaerobic chemostat cultures have shown that there is a significant flux to 2-oxoglutarate also under anaerobic conditions (Nissen et al., 1997). Apart from the mitochondrial pool of 2-oxoglutarte there is a cytosolic one as well. Parts of this could come via transportation from the mitochondrial pool via one of the two known mitochondrial transporters of 2-oxoglutarate; Odc1p and Odc2p (Palmieri et al., 2000). There are further cytosolic iso-enzymes to those of the TCA-cycle, enabling direct cytosolic formation of 2-oxoglutarate (Fig. 8). This is of importance as we throughout our studies have been expressing EFE cytosolically and cytosolic availability of 2-oxoglutarate is therefore required. Mitochondrial localization of the EFE has been tried via addition of a localization tag at the N-terminal, however no ethylene formation was seen with this construct (Pirkov et al., 2008).

2.3 Respiration and/or fermentation

As seen from the above *S. cerevisiae* is a facultative anaerobe, i.e. it respires in the presence of oxygen and ferments if oxygen is absent. *S. cerevisiae* further belongs to the so-called Crabtree positive yeasts (De Deken, 1966) as it will ferment even in the presence of oxygen under certain conditions, giving a respiro-fermentative growth (Crabtree, 1929). This mixed type of growth will be observed when the external glucose level exceeds 0.8 mM (Verduyn et al., 1984) and when growth rates are high. It has the biological advantage of increased energy production rate compared to either of the two “pure” growth versions (Käppeli, 1986). Under conditions were the cells are suddenly exposed to a sharp increase in glucose levels overflow ethanol formation in a short-term version of the Crabtree effect can also be seen (Pronk et al., 1996). The metabolic explanation for the phenomenon of fermentation also under aerobic conditions can be found within the capacity and regulation of the central carbon metabolism pathways of the yeast.

2.3.1 Regulation of the central carbon metabolism

The underlying reason for the overflow metabolism of aerobic fermentation has been studied intensely through the years. Early studies indicated that the onset of the Crabtree effect was due to limitations in respiration capacity (Rieger et al., 1983b). This hypothesis was strengthen by experiments showing that expression of an alternative heterologous oxidase resulted in lower aerobic ethanol levels (Vemuri et al., 2007). It has been reasoned that Crabtree negative yeast have higher respiratory capacity and hence therefor no overflow metabolism, Verduyn et al. (1992) however showed that at low dilution rates *S. cerevisiae* could attain oxygen consumption rates even higher than those measured for Crabtree negative yeast when the uncoupler benzoic acid was added to the media, thus contradicting the claim somewhat.

Enzymes at the pyruvate branch-point have also been specified as central to the onset of aerobic ethanol formation (Pronk et al., 1996). It was shown that the activity of pyruvate decarboxylase is higher in a Crabtree positive yeast than in negative ones under glucose excess, while the activities of acetaldehyde dehydrogenase and acetyl-CoA synthetase are higher in Crabtree negative yeasts indicating that the so called pyruvate dehydrogenase by-pass is more active in Crabtree negative yeasts. This would thus give that in Crabtree negative yeasts excess acetaldehyde is effectively oxidized to acetyl-CoA while in e.g. *S. cerevisiae* it has to be reduced to ethanol (van Urk et al., 1989).

Another factor influencing the onset of aerobic fermentation is glucose governed down regulation of the respiratory metabolism (often known as glucose repression) (see eg. Carlson, 1999; Cortassa and Aon, 1998). Heyland et al. (2009) reported that in batch cultivations the flux through the TCA cycle is inversely correlated to the specific glucose uptake rate. Correlating to the previous finding that the higher the glucose uptake capacity of the glucose transporter is, the higher the ethanol production is (Elbing et al., 2004). Further, limiting the glucose uptake capacity of the yeast can eliminate aerobic fermentation almost completely even at high external glucose levels (Otterstedt et al., 2004) indicating a regulatory function of glucose on the central carbon metabolism. It has been suggested that it is the glucose consumption rate which is the trigger signal for this gene regulation (Reifenberger et al., 1997).

2.4 Nitrogen metabolism

The nitrogen metabolism is made up of an intrinsic network of metabolic reactions, at the center of which are the two amino acids glutamate and glutamine (Cooper, 1982; Holmes et al., 1991). The two are inter-convertible (Fig. 9); glutamate is aminated into glutamine via the ATP dependent glutamine synthase (GLN1) (Mitchell and Magasanik, 1983), whereas glutamine can be converted to glutamate via the NADH-dependent glutamate synthase (GOGAT or GLT1) (Cogoni et al., 1995).

The commonly used nitrogen source ammonia is assimilated via glutamate, through a reaction with 2-oxoglutarate catalyzed by the two enzymes NADPH-dependent glutamate dehydrogenase 1 and 3 (GDH1 and GDH3) (Avendano et al., 1997; DeLuna et al., 2001; Nagasu and Hall, 1985). The metabolite 2-oxoglutarate is hence not only an important intermediate of the TCA cycle (and one of the main substrates of the EFE), it also plays a significant role in the nitrogen metabolism of *S. cerevisiae*. Ammonia is also required by GLN1 to convert glutamate into glutamine (Fig. 9). It has been found that in nitrogen starved cells supplied ammonium will initially be assimilated into only glutamine and glutamate before the nitrogen is redirect throughout the cell metabolism (Holmes et al., 1991). It has further been found that independent of nitrogen source utilized a relatively large pool of glutamate will be kept intracellularly indicating its important and central role (Watson, 1976). In the end approximately 85 % of all incorporated nitrogen of the cell will have come via glutamate while the remaining 15 % is derived from glutamine (Cooper, 1982). The formation of amino acids as well as nucleotides is dependent on the two either as precursors or as amine donors in so called transamination reactions. (Jones and Fink, 1982; Ljungdahl and Daignan-Fornier, 2012). In transamination reactions with glutamate as donor 2-oxoglutarate will be formed as a side product. It has been shown that in anaerobic cultivations grown on glutamate 2-oxoglutarate levels are substantially increased (Albers et al., 1998; Albers et al., 1996; Lewis and Rainbow, 1963). We have observed the same for aerobic cultivations (**paper II and III**) (Fig. 11B). This is likely due to the effect of transamination reactions (Lewis and Rainbow, 1963). Another effect of utilizing glutamate as nitrogen source is a large increase in biomass as compared to cells grown on ammonia, as the carbon backbone of the glutamate is shunted into biomass formation.

2.4.1 Selected amino acid metabolism

One of the glutamate derived amino acids central to this work is the EFE substrate/co-factor arginine. In short biosynthesis of arginine is a multi-step process where glutamate is first converted to ornithine via several enzymatic steps. The ornithine is combined with carbamoyl-phosphate in the first step of the urea cycle to form citruline which is subsequently converted into arginine via two enzymatic steps (Jauniaux et al., 1978)(Fig. 9). We have investigated the effect of using arginine as nitrogen source, either on its own (**paper III**) or in combination with glutamate (**paper II and III**). We found that when arginine was used as the sole nitrogen source large amounts of pyruvate were produced (**paper III**), interestingly the same has been found when glycine was used as nitrogen source, but is not seen when ammonia or glutamate is utilized (Albers, 2000). Indicating that the carbon backbone of some amino acids can feed into pyruvate formation and the central carbon metabolism, or has a regulatory function on the carbon metabolism.

Another amino acid whose metabolism is worth a short discussion is proline. Like arginine, proline is formed from glutamate (Fig. 9). The pathway goes via the metabolite P5C, which is also a product of EFE. The catabolism of proline will render glutamate again. Interestingly the first step in the catabolic metabolism is the re-conversion of proline into P5C again. To avoid a futile circle between proline and P5C the anabolic and catabolic pathway are compartmentalized to the cytosol and mitochondria respectively (Brandriss and Magasanik, 1980; Brandriss and Magasanik, 1981; Cooper, 1982). Larsson et al. (2011) suggested that adding external proline might increase ethylene yields as *in silico* results showed that when proline flux was allowed to vary the predicted ethylene yield increased 65 % due to an increased flux to glutamate. The model did however not take the localization of the different pools into account.

It is obvious that the central carbon and nitrogen metabolisms are highly interlinked and dependent on each other, especially for amino acid and nucleotide formation. The question is how coordinated are the two and what governs the coordination? It has been put forward that in *E.coli* 2-oxoglutarate is the coordinating metabolite, down-regulating carbon metabolism via competitive inhibition of the citrate synthase and 3-phosphoglycerate dehydrogenase when nitrogen becomes limiting (Doucette et al., 2011). Perhaps the same function is true in *S. cerevisiae*, where 2-oxoglutarate is also found in the intersection of the central carbon and nitrogen metabolisms.

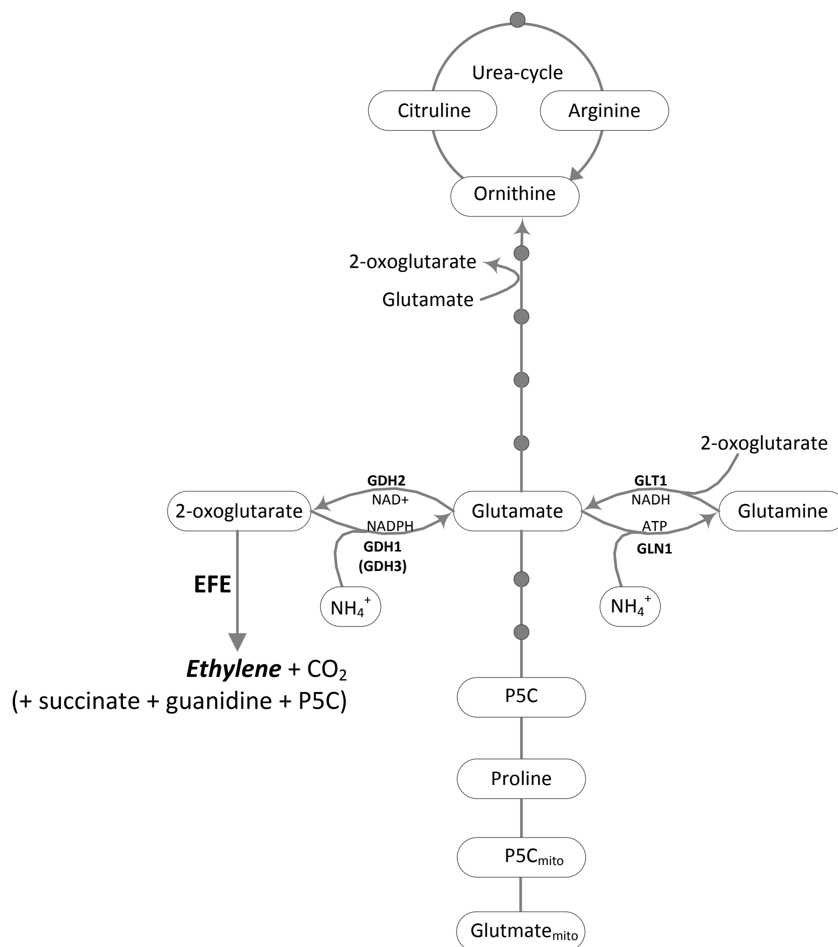


Figure 9. Selected parts of the nitrogen metabolism of *S. cerevisiae*
showing the intimate link between the EFE substrate 2-oxoglutarate and the central amino acids glutamate and glutamine. Indicated in the figure is also the pathway to the EFE substrate/co-factor arginine and the EFE product P5C, which is part of the proline metbaolism

2.5 Preferred nitrogen source utilization

S. cerevisiae can support growth on a multitude of nitrogen source apart from ammonia, including urea, allantoin, γ-aminobutyrate (GABA) and all L-amino acids except lysine, histidine and cysteine. However, just like glucose is a preferred carbon source certain nitrogen sources are preferred over others. Comparing the generation times of *S. cerevisiae* grown on different nitrogen sources (with glucose as carbon source), good nitrogen sources will support generation times around 2 hours, whereas poorer ones can result in generation times well above 4 hours (Cooper, 1982; Niederberger et al., 1981).

As second factor, apart from growth rate, which is commonly take into account when ranking nitrogen sources into good/poor is their effect on the utilization of other nitrogen sources present. So called nitrogen catabolite repression (NCR) is the cells way of regulating the nitrogen source used to the best option available. A good nitrogen source will inhibit the transcription activators Gln3 and Gat1 required for uptake and degradation of poorer nitrogen sources, thereby minimizing the need for expression of excess catabolic routs. In the absence of a rich nitrogen source, de-repression of Gln3 and Gat1 will occur and expression of required metabolic genes is initiated (Cooper, 2002; Hofman-Bang, 1999). Generally it is said that the stronger the NCR effect the more preferred as a nitrogen source the compound is.

The actual classification of different nitrogen sources into preferred and non-preferred does however not seem to be as straight forward as one could believe, and as some suggest, as different studies group the possible nitrogen sources differently (Godard et al., 2007; Magasanik and Kaiser, 2002). It has further been noticed that some strain variability does exist (Magasanik and Kaiser, 2002; Rytka, 1975). One of the amino acids which vary in classification dependent on study is the EFE substrate/co-factor arginine. Godard et al. (2007) did an extensive study on the effect of 21 different nitrogen sources on the gene expression, with special focus on genes connected to NCR. They found that short generation times were linked to a clear NCR effect. They classified ammonia, glutamate as well as arginine as preferred nitrogen sources though arginine was found to support slightly slower growth.

What has been noticed is that many good nitrogen sources upon either deamination or transamination will give compounds found within the central carbon metabolism (Godard et al., 2007), which is true for e.g. arginine glutamate and glutamine, whereas non-preferred ones often give non-usable fusel acids and alcohols (Hazelwood et al., 2008). Some good nitrogen sources also easily connect to the central nitrogen metabolism and are hence quickly and effortlessly utilized by the cell as is the case with ammonia.

Though several compounds are classified as preferred, there is a hierarchy also in-between these. We saw that in batch cultivations where glutamate and arginine were used in combination as nitrogen source the uptake of arginine was delayed until the available glutamate was spent (**paper III**) (Fig. 10). This correlates with previous findings that most amino acids will be used sequentially and that glutamate so to say trumps arginine as it is an early consumed nitrogen source whereas arginine is a later consumed one (Crepin et al., 2012).

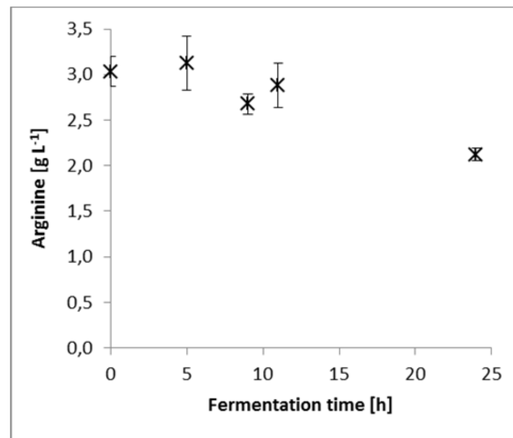


Figure 10. Arginine uptake in mixed nitrogen source cultivations
Extracellular concentration of arginine in batch culture with
3.5 g L^{-1} glutamate and 3.5 g L^{-1} arginine as nitrogen sources.

2.6 Growth in batch vs chemostat

Bioreactors can be operated in several different ways; batch, fed-batch and chemostat, giving different growth patterns and physiology of the cultivated yeast. In a batch culture the system is closed in the sense that after inoculation (i.e. addition of yeast) no extra media is added to the system. In aerobic cultures on glucose this will give a so called diauxic growth pattern for *S. cerevisiae*. Initially when the glucose levels are high respiro-fermentative growth with ethanol formation will occur due to the Crabtree effect, when glucose is spent the cells will go through an adaptive phase, the diauxic shift, and switch to purely respirative growth based on the ethanol formed in the first phase of the cultivation. A thorough study of the diauxic shift revealed that the readjustment of the metabolism will occur well before all glucose is spent with reduced glycolytic flux and increased flux towards storage molecules occurring (Zampar et al., 2013). A batch-type of set-up hence has a clear beginning and end, is easy to set up and productivity of the sought after product can be followed for different physiologic stages of the yeast.

In contrast in a chemostat setting media is pumped into the vessel at a set dilution rate, while culture is pumped out of the system at the same rate. This gives the culture a constant volume and at the same time a controlled environment and constant growth rate governed by the dilution rate, the culture enters a so called steady state. The yeast will grow purely respirativly at low dilution rates, however as the dilution rate is increased the cells will at one point switch to respiro-fermentative growth and start to form ethanol. Usually this will occur at dilution rates between 0.25 and 0.3 h^{-1} (Frick and Wittmann, 2005; Van Hoek et al., 1998). However, it has been shown that the respiro-fermentative growth can be prompted early or that an

extended period of respiro-fermentation can take place, if the increase in dilution rate is done at to large increments (Postma et al., 1989; Rieger et al., 1983b). Finally a dilution rate is reached at which the yeast is unable to maintain the growth rate required and it will subsequently be washed out of the bioreactor. The main advantage of this type of culture is that it allows for direct correlation between physiological state of the cell and selected environment conditions as the steady state is time-independent. Chemostat cultures were hence classically used to study fundamental issues.

A third version (which has not been applied in our studies) is the fed-batch system, in some ways a mix of the two previous ones. In this setup fresh media or substrate is added to the system either continuously or pulse wise, but no culture is withdrawn. The volume of the culture will hence increase throughout the cultivation (there are also methods where the limiting growth factor is added without increasing the volume, e.g. through dialysis). This cultivation type can give high biomass concentrations as well as controlled growth rates, however accumulations of toxins could in some circumstances become an issue.

2.7 Ethylene toxicity and transportation

An issue which always has to be addressed in biotechnical production of chemicals is the toxicity of the product for the host microorganism. In general it has been observed that unsaturated gases are more growth inhibiting than corresponding saturated gas and that the more hydrophobic the gas is the higher is its toxicity (Kawachi et al., 2010). Extracellularly applied ethylene has also been suggested to reduce the glucose uptake rate of *S. cerevisiae* (Thomas and Spencer, 1978) and high extracellular pressure of ethylene leads to invagination of the yeast cells (Kawachi et al., 2010). With that said, the IP_{50} (pressure where growth is inhibited by 50 %) of ethylene on *S. cerevisiae* was found to be 0.607 MPa (i.e. 6 atm) (Kawachi et al., 2010), which is several magnitudes of order higher than what we have achieved in our cultivations. Another issue which could become problematic for the production is intracellular accumulation of the product, both due to toxicity and from a processing point of view. No membrane transporters linked to ethylene has been reported, however as small uncharged gaseous molecules such as oxygen and carbon dioxide pass through the membrane via diffusion it is very likely that ethylene, also being small and uncharged, likewise passes through the cell membrane through diffusion.

.

2.8 Ethylene production in bioreactors

All of our bioreactor cultivations were set up in 3.5 L Belach (Belach Bioteknik AB, Skogås, Sweden) bioreactors. The off gas of this system was led via a pumping system and overflow release system directly to a GC-FID thus enabling on-line and real-time measurements of ethylene concentrations. As the ethylene levels measured are real-time values and given as production rates (production per hour), the total production of a batch cultivation will be given by the area under the curve in a rate-time graph. Determination of this area can easily be achieved by employing the trapz(x,y) function in MatLab (MathWorks, USA). One concern for the measurements is the solubility of ethylene in the culture broth. It has been determined that solubility of ethylene in culture media has an Ostwald coefficient (mL of gas per mL of liquid) of 0.11 at 30˚C (Serra et al., 2011). However as the bioreactor has a constant air flow through it a gas stripping effect will occur, something which has been proven effective for extraction of ethylene from liquid (Bassi et al., 1981). We have further determined experimentally that the stripping effect was independent of dilution rate (**unpublished data**).

2.8.1 Ethylene sampling in different culture set-ups

Quick evaluations of different strains for ethylene production can be performed by employing shake flask cultures. As these reach an OD of approximately 1.0 the regular gas passing plug is exchanged for a rubber plug with a syringe needle through it. The culture is incubated for a further 30 min, to accumulate gas, after which a head space sample is extracted via the needle using a syringe. The gas sample is subsequently directly injected into the GC-FID. This was the system used in the evaluation of mutations of the EFE presented in previous chapter.

Evaluation of the effect of metabolism on ethylene formation was however performed in bioreactors to enable increased control of the cultivation conditions. In batch cultivations the off-gas was in general sampled every 30 minutes through-out the cultivation. For chemostat cultivations samples were only taken at more disperse time points during the start-up. As steady state was approached sampling was switched to every 30 min until a steady ethylene level was seen and kept at this frequency throughout the sampling.

2.9 Effect of nitrogen source on ethylene production

One of the factors we have studied extensively is the effect of nitrogen source on ethylene formation. Experiments have been performed in batch as well as chemostat cultivations. The effect was initially investigated in chemostat (**paper II**), and like previous studies (Larsson et al., 2011; Pirkov et al., 2008) changing the nitrogen source from ammonia to glutamate led to an almost 50 % increase in ethylene productivity (µg L^{-1} h^{-1}). The specific ethylene productivity (µg g_{DW}^{-1} h^{-1}) was however similar for the two conditions as the biomass concentration was also increased considerably with glutamate (Table 2). In follow-up batch cultivations (**paper III**) some distinct differences was observed between cultures on glutamate and the reference cultures with ammonia. The ethylene productivity of the ammonia based cultivations peaked during the late glucose phase, whereas for the glutamate cultures the maximal productivity was reached during the later parts of the ethanol phase.

Table 2. Effect of nitrogen source on ethylene formation in chemostat cultivations

	Ethylene			
N-Source	Biomass [g L^{-1}]	Productivity [µg $L_{Culture}^{-1}$ h^{-1}]	Specific productivity [µg g_{DW}^{-1} h^{-1}]	Yield [µg $g_{Glucose}^{-1}$]
$(NH_4)_2SO_4$ (7.5 g/L)	5.72 ± 0.95	178 ± 25	30.4 ± 2.8	164 ± 21
Glutamate (7.5 g/L)	7.48 ± 0.14	242 ± 2	32.3 ± 0.3	233 ± 0.8
Glutamate + Arginine (3.5 g/L each)	7.34 ± 0.03	101 ± 1	13.8 ± 0.2	96.8 ± 1.0

Production data and biomass formation when three different nitrogen sources are employed in chemostat cultivations with 10 g glucose L^{-1}. All cultivations performed at at D=0.1 h^{-1}.
± Minimal and maximal ethylene production levels

Comparing the specific ethylene productivities of the two conditions it became obvious that with glutamate a relatively high and stable ethylene level was kept also in the ethanol phase, whereas with ammonia the level was declining and much lower (Fig. 11a and b – upper graph). Studies of extracellular metabolites determined that the levels of 2-oxoglutarate were more than 10 times higher in the ethanol phase of the glutamate cultures compared to those of the ammonia cultures (Fig. 11a and b – lower graph). Hence, there is a considerably increased availability of the substrate 2-oxogltuarate for the EFE in glutamate cultures, especially during the second respirative phase, explaining the higher ethylene levels reached in these cultures.

The EFE substrate/co-factor arginine has been proposed to be a limiting metabolite within glucose controlled continuous cultures of *S. cerevisiae* (Boer et al., 2010). It was hence investigated if arginine could be a restricting factor for ethylene formation in chemostat cultivations by replacing parts of the glutamate with arginine as nitrogen source (**paper II**). Surprisingly it was found that in these cultures the ethylene levels were severely reduced (Table 2). Subsequent batch cultivations on arginine as sole nitrogen source confirmed that ethylene levels were negatively affected when using arginine (Fig. 11c). Contradictory to in the situation in the chemostat, batch cultures on a mixture of glutamate and arginine did not show reduced ethylene levels, but rather showed levels similar to those on purely glutamate (**paper III**) (Fig. 11d). For the mixed batch cultivations it was shown that arginine, as a poorer nitrogen source than glutamate, was only consumed after the glutamate was spent late in the experiment (Fig. 10). Calculating the nitrogen requirements of the biomass formed in the mixed chemostat samples, it was concluded that co-consumption of the nitrogen sources were necessary to reach the requirements. It hence seems that the intracellular concentration or the metabolism of arginine has a negative effect on the ethylene formation via EFE. Strategies to clarify and get around this issue is further discussed in **chapter 3**

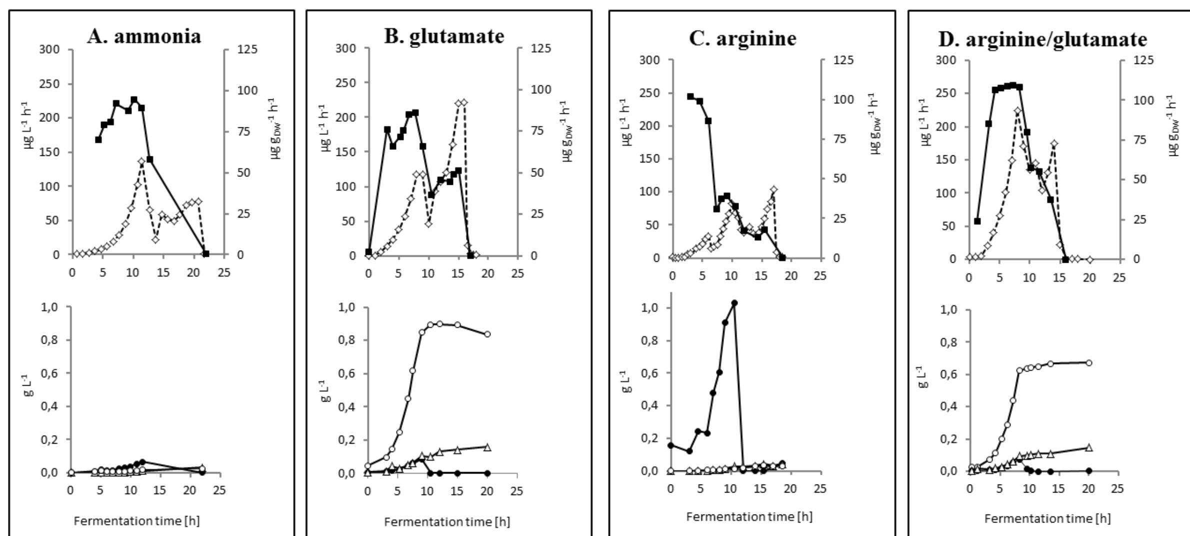


Figure 11. Production pattern in batch cultures depending on nitrogen source
In cultures with 10 g glucose L^{-1} and either 7.5 g L^{-1} ammonia (A), glutamate (B) or arginine (C) or 3.5 g L^{-1} glutamate in combination with 3.5 g L^{-1} arginine (D).
Upper graphs: ethylene productivity (open diamonds) and specific ethylene productivity (solid squares).
Lower graphs: 2-oxoglutarate (open circles), pyruvate (closed circles), succinate (open triangles)

2.10 Influence of growth and respiration rate on ethylene formation

Variations in growth rate are linked to genetic and metabolic differences (Fraenkel, 2011; Regenberg et al., 2006). As the growth rate can be controlled via the dilution rate in a chemostat, ethylene production was determined at different dilution rates varying from 0.033 h^{-1} to 0.35 h^{-1}, thus spanning from pure respirative growth to mixed respiro-fermentative (**paper II**). The specific ethylene productivity (µg g_{DW}^{-1} h^{-1}, closed circles in Fig. 12) increased throughout the dilution span, though not in a linear fashion. Between dilution rates 0.15 h^{-1} and 0.25 h^{-1} a lower increases in specific productivity was seen with the dilution rate increases. Between dilution rates 0.25 h^{-1} and 0.3 h^{-1} a big jump in specific productivity is seen, this correlates with the shift to respiro-fermentative growth, which can further be seen on the onset of ethanol formation and on the typical overflow of pyruvate from the glycolysis as well as increased acetate formation and reduced biomass formation (Fig 12b and c). The opposite trend to that of the specific productivity was seen for the yield, which decreased with each increase in dilution rate.

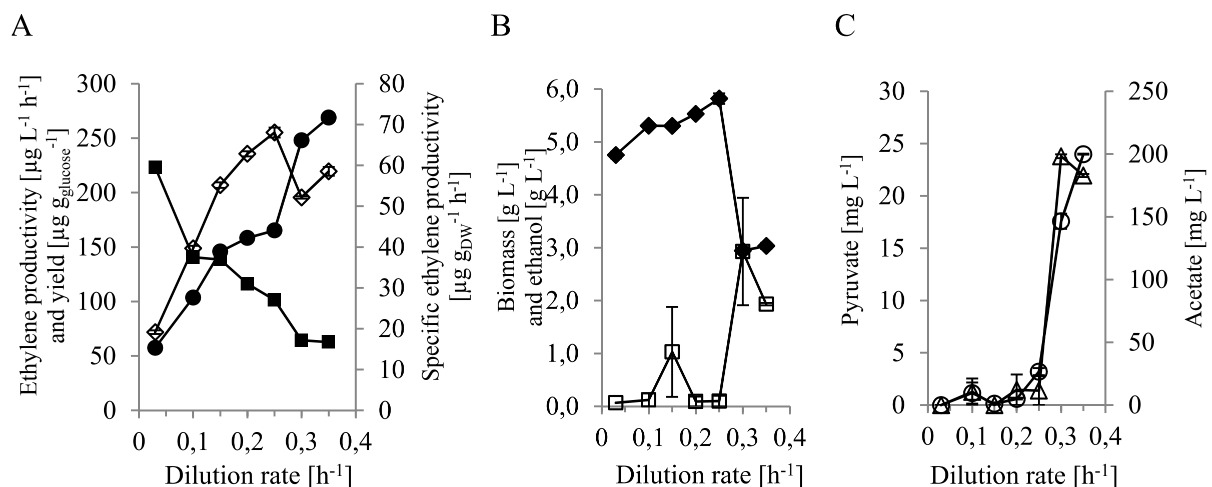


Figure 12. Growth and product characteristics with increasing dilution rate
A: Ethylene productivity (open diamond), specific productivity (closed circle) and yield (closed square).
B: Biomass (closed diamond) and ethanol (open square). C: Pyruvate (open circle) and acetate (open triangle).

Through metabolic modeling it was suggested that respiration rate and NADH re-oxidation could be a limiting factor for ethylene formation (Larsson et al., 2011). As mentioned previously the respiration rated could be increased via the addition benzoic acid to the media (Verduyn et al., 1992). We saw that the addition of 7.5 mM benzoic acid had a positive effect on specific ethylene production (μg g_{DW}^{-1} h^{-1}), increasing it 65 % compared to the standard condition without addition (Table 3). When the benzoic acid enters the cell it will dissociate and thus disturb the cytosolic pH and possibly also membrane organization (Hazan et al., 2004), the acid is therefore pumped out of the cell via a plasma membrane ATPase (Holyoak et al., 1999). The increased usage of ATP is met by an increase in respiration rate. However, as a large part of ATP is used for the pumping, a significant decrease in biomass follows as an effect of benzoic acids addition (Warth, 1988; Verduyn et al., 1992). This was also seen in our cultures and hence the productivity (μg L^{-1} h^{-1}) was low (**paper II**).

To further examine the coupling between ethylene formation to the respiration rate, the effect of blocking the respiration via addition of the cytochrome c oxidase inhibitor sodium azide (Duncan and Mackler, 1966) was investigated. Addition completely abolished ethylene formation, supporting the hypothesis that ethylene formation is intimately linked to the respiration and that increasing respiration could be one step in achieving higher ethylene production levels, more on this will follow in **chapter 3**.

Table 3. Effect of respiration rate on ethylene formation

	Ethylene	
Condition	Specific productivity [μg g_{DW}^{-1} h^{-1}]	Productivity [μg $L_{Culture}^{-1}$ h^{-1}]
Reference cond.	30.4 ± 2.8	178 ± 25
+ 7.5 mM Benzoate	50.3 ± 1.3	37.7 ± 0.9
+ 1 mM Azide	0	0

Benzoic acid was added in order to increase respiration rate, while azide was added to block the respiratory chain. Experiments were performed in chemostat cultivations operated at $D=0.1h^{-1}$ and with 10 g glucose L^{-1}.

2.11 Some additional factors evaluated

A few other factors apart from the ones mentioned above have also been evaluated for their effect on ethylene formation. None of these results have been included in the manuscripts I, however, still thought that the results were worth mentioning here. One factor which was investigated was the effect of cultivation temperature on ethylene formation. The temperature optima of the *P. syringae* EFE has been determined to be 20-25˚C (see table 1), at temperatures above this increased inclusion body formation of the enzyme was seen in *E. coli* (Ishihara et al., 1995). Batch cultivation of our *S. cerevisiae* strain at 25˚C however only led to slower growth and decreased specific productivity.

Heyland et al. (2009) showed that employing sub-optimal pH (3.2 and 6.9) led to increased TCA cycle flux. As ethylene formation is linked to the TCA intermediate 2-oxoglutarate, increased TCA flux could possibly positively influence the ethylene formation, thus batch cultivations were performed at pH 3.5 and 7.0. These showed an increase in production rates as well as yields. However, when the same was tried in a chemostat the effect was not replicable. There pH 3.5 still gave a higher ethylene productivity, while at pH 7.0 ethylene formation was substantially reduced. The effect of pH on ethylene formation is hence inconclusive.

From the experiments described it this chapter several factors have been identified as possible targets for improved ethylene formation. Intracellular arginine concentration seems to function negatively on ethylene formation whereas increased availability of the substrate 2-oxoglutarate seems to have a positive effect. It is further obvious that the respiration rate of the host is central for improved ethylene formation. The next chapter covers how these points could be met via metabolic engineering of the host.

CHAPTER 3

METABOLIC IMPROVEMENTS OF THE S. CEREVISIAE-EFE CELL FACTORY

In the early 1900's the first industrial processes using pure microbial cultures for controlled production of chemicals emerged, with the production of acetone using the bacterium *Clostridium acetobutylicum* (1916) and citric acids production using the fungus *Aspergillus niger* (1923) (Springham et al., 1999). Through the past century this microorganism based production has expanded and a multitude of bio-based chemicals are industrially available or under development today including different biofuels, bulk and fine chemicals, polymers or their building blocks and pharmaceutical compounds (for reviews see e.g. Hong and Nielsen, 2012; Otero and Nielsen, 2010). Central for the progress of this field is strain development, in order to expand both the product and substrate range as well as to increase the yields, titers and rates of production of the microbial producers in place today (Buschke et al., 2013; Tyo et al., 2007). In this chapter I will review how these strain developments can be guided and implemented. I present how metabolic targets for improved production of ethylene in *S. cerevisiae* were identified and the effects they had on *in vivo* ethylene production.

3.1 Metabolic engineering

The first microorganism based chemical production set-ups were developed through screening of strains for native production abilities and choosing the best (or only) producer of these. Quickly the idea of improving the productivity through alterations of the original strain grew strong. This was mainly achieved through application of chemical mutagens and screening for an increase in production (Stephanopoulos et al., 1998). The knowledge of the underlying genetic and metabolic alteration giving rise to the change was hence very limited. The technique was however still relatively successful in certain instances such as for penicillin production (Nielsen, 1995; Ågren, 2013). With the determination of the structure of DNA (Watson and Crick, 1953) and the understanding of the molecular basis underlying the central dogma (i.e. DNA→RNA→Protein) (Crick, 1970) methods for more directed alterations were being developed. In the 1970's the first so called genetically engineered organisms were created and in the following decades the molecular biology toolbox for performing directed genetic modifications grew. Various terms were used to describe this area, the persistent one became 'metabolic engineering' (Bailey, 1991; Stephanopoulos and Vallino, 1991) and has

been defined as "*the directed improvement of product formation or cellular properties through the modification of specific biochemical reaction(s) or the introduction of new one(s) with the use of recombinant DNA technology*" (Stephanopoulos et al., 1998). This is today a central area within biotechnology.

3.1.1 Impact of rewiring of the nitrogen metabolism on ethylene formation

In **paper II** it was found that high levels of arginine had a detrimental effect on ethylene formation (as discussed in **chapter 2**), in **paper III** metabolic engineering of the arginine metabolism was used to try to meet this finding. In an attempt to understand the underlying reason for the effect and to see if reduced arginine availability could improve ethylene formation two different metabolic strategies were developed and implemented. In order to increase the degradation of arginine, overexpression of the arginine catabolic enzyme Car1, which catalyzes the reaction from arginine to ornithine (Middelhoven, 1964) (Fig. 13), was tried. This had only a minor effect on ethylene formation, 84.0 ± 10.7 µg $g_{glucose}^{-1}$ compared to 75.4 ± 18.7 for the reference strain with ammonia as nitrogen source (**paper III**). Further investigations into the arginine metabolism reviled that the next step in the arginine degradation pathway, catalyzed by Car2, is 2-oxoglutarate dependent (Middelhoven, 1964). This could be the underlying reason for why increased concentrations of arginine reduce ethylene formation. It resulted in depletion of the EFE substrate, rather than the initial hypothesis of paper I that arginine availability shifts the ration between the two reactions of the EFE in favor of the non-ethylene forming reaction, for which a larger effect of the Car1overexpression would have been expected.

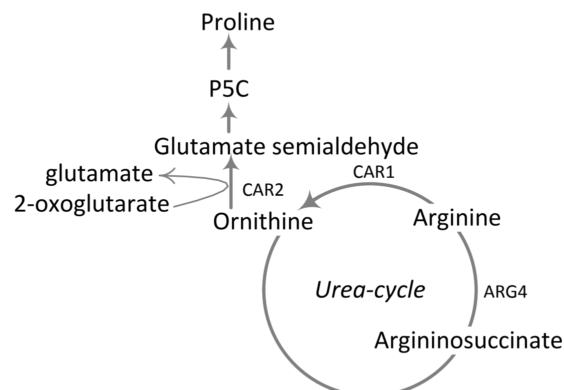


Figure 13. Arginine metabolism in *S. cerevisiae*
indicating the 2-oxoglutarate dependent step in the catabolism catalyzed by Car2.
Arg4: Argininosuccinate lyase, Car1: Arginase, Car2: L-ornithine transaminase,
P5C: 1-Pyrroline-5-carboxylic acid.

If it is the metabolism of arginine which is influencing, a minimization of the arginine availability to only cover the basic requirements could hence have a postive effect on ethylene formation. To investigate this hypothesis, a Δ*arg4* strain was contructed. This gives a strain which should be unable to grow without the addition of arginine to the media as Arg4 catalyzes the final step in the arginine anobolic pathway (Fig. 13). An evaluation of the arginine requirment of the Δ*arg4* strain was thus perferomed via addition of a wide span of arginine concentrations to shake flask cultivations and measuring the final OD of the cultures. When no arginine was added the strain did not grow at all, showing that the deletion of *ARG4* was effective in restricting the arginien formation, while addition of 1 mM arginine led to normal growth. As ethylene production peaks at late glucose phase, arginine concentrations which would lead to an arrest in growth before the diauxic shift were therefore chosen for further tests in bioreactors. However, no increase in ethylene formation could be seen as arginine concentration became low, rather there was a rapid decrease in ethylene formation even though there was still glucose present and biomass formation continued (Fig. 14). Hence, it has to be concluded that a fine balancing of the available argininge is required for optimal ethylene production (**paper III**).

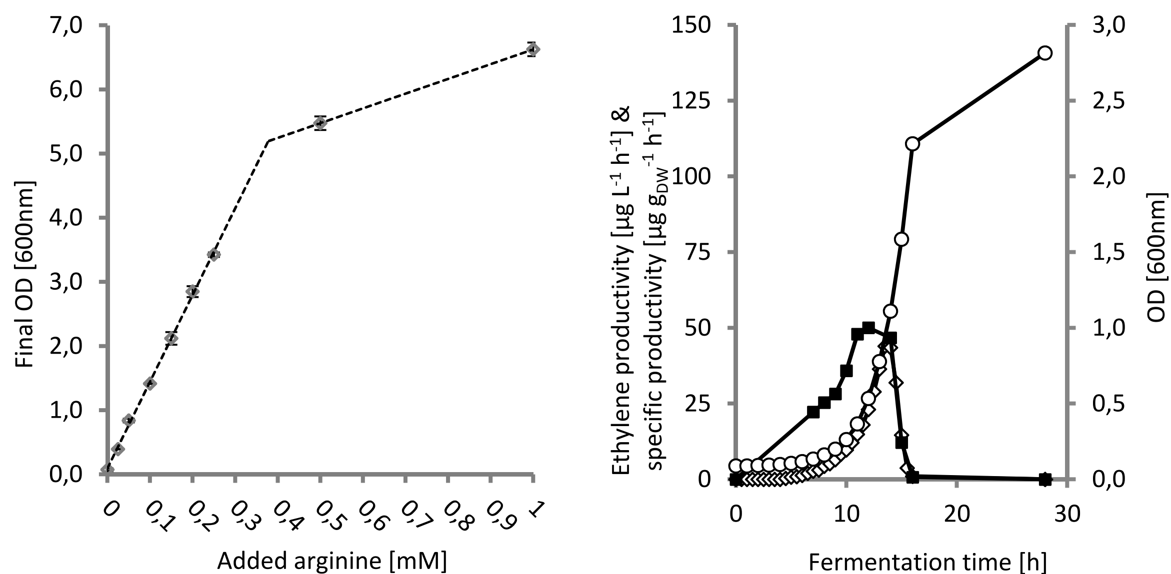


Figure 14. Effect of arginine on OD and ethylene production in a *Δarg4* strain
Left) Final OD of the shake flask cultures of the *Δarg4* strain with varying amounts of arginine added.
Right) Ethylene productivity (open diamonds), specific ethylene productivity (closed squares) and OD (open circles) of batch cultivation of the *Δarg4* strain with 2 mM arginine addition and 2% glucose.

3.2 Metabolic modeling

Even though there is a large tool box for performing metabolic engineering and a vast biochemical knowledge has been accumulated through the years the technique can still be limited by the complexity of the organism's metabolism. Interconnectivity together with co-factor and re-dox balancing means that getting a complete overview and understanding of a biological system is hard. To get a more holistic view, scientists started to put together extensive maps of the metabolic networks and network reconstruction became an important tool in metabolic engineering. Simple metabolic network models of first *E. coli* (Varma et al., 1993) and subsequently also for *S. cerevisiae* (van Gulik and Heijnen, 1995) were published in the mid 90's. A few years later the breakthrough of genome sequencing laid the foundation for so called genome scale metabolic models.

The construction and evaluation of a genome scale metabolic model is a major, iterative work, the starting point of which is the reconstruction of the metabolic network based on gene annotation and biochemical knowledge. When a draft version of the network is pieced together, which links genes to reactions and metabolites, it has to be curated, removing falsely identified genes and gene products. This is to a large extent a manual work. Co-factor usage, reaction stoichiometry and directionality have to be examined for each reaction and compartmentalization as well as transportation taken into account (Thiele and Palsson, 2010; Österlund et al., 2012). Once this is done the information is converted into a mathematical model by translating the reactions from the reaction network into mass balance equations. Here the assumptions are often made that the effect of growth correlated volume increase is neglectable (as shown by Zupke and Stephanopoulos, 1995) and that the concentration of intracellular metabolites is at a steady state and does not change over time (as discussed by Varma and Palsson, 1994). The complete set of mass balance equations can then be represented by a stoichiometric matric (S) and a flux vector (v) as $S \bullet v = 0$ (the stoichiometric matrix is composed with reactions as columns and metabolites as rows, a substrate has a negative coefficient while a product has a positive one). Boundaries are set to the system by defining exchange reactions of metabolites consumed and excreted by the cell. Finally constraints are set to reduce the number of possible solutions for the system (Thiele and Palsson, 2010).

Once a genome scale metabolic model is worked out, it has to be quality controlled and validated. This can for example be done by comparing predicted results from the model to available experimentally observed values (Duarte et al., 2004). Usually this reveals some discrepancies between model and reality and the iterative process takes place where faults in the model, such as dead end or orphan reactions, unbalanced charges and inconsistencies in stoichiometry, are identified and corrected followed by a new prediction (Thiele and Palsson, 2010; Österlund et al., 2012).

When a model is in place it can then be used for many different applications. Österlund et al. (2012) defined four major areas; i) guidance for metabolic engineering – this is the application area used for our research (**paper IV**), ii) biological interpretation and discovery – to help analyze and understand omics data, iii) application of novel computational framework – through which several different methods for modeling has been develop e.g. Flux variability analysis (FVA) (Mahadevan and Schilling, 2003) as used in **paper IV**, and iv) evolutionary elucidation – helping identify preserved pathways (Vitkup et al., 2006) and evaluating the effect of gene duplication (Kuepfer et al., 2005) etc..

The benefit of using metabolic modeling obviously is the ability to view the complete system at once. This way issues, solutions and effects which could not be seen by looking at only the specific pathway can be identified. Comparing to the traditional rational (or reductive) way of identifying metabolic engineering targets, metabolic modeling gives a much more holistic view and can hence help drive development and understanding further, faster.

3.2.1 S. cerevisiae models

As developing a metabolic model is an iterative process the work with them can be almost endless. Often it is the aim of the project or the objective functionality of the model which defines when it is done. Hence, several genome scale metabolic models of *S. cerevisiae* have been developed through the years. The first version (iFF708) was released in 2003 by Förster et al. (2003) and contained 1145 reactions. However a number of the included reactions were dead end ones and the model was hence developed further during the following decade e.g.; iLL672 (Kuepfer et al., 2005), iND750 (Duarte et al., 2004), iMH805/775 (Herrgård et al., 2006), iIN800 (Nookaew et al., 2008) and iMM904 (Mo et al., 2009). However, as different modelers used different modelling approaches there were discrepancies between the models and it was decided within the community to form a consensus model. The first version of this (Yeast 1.0) was thus released in 2008 (Herrgård et al., 2008). Some of these genome scale

metabolic models have since been used successfully to identify metabolic strategies for increase product of bioethanol (Bro et al., 2006) and sesquiterpene (Asadollahi et al., 2009). Metabolic modelling has also been used to compare the plant and EFE pathways for ethylene production in *S. cerevisiae* (Larsson et al., 2011). This model was however only based on the central carbon metabolism of the yeast and no compartmentalization was taken into account, therefore a full genome scale metabolic model of the EFE-*S. cerevisiae* cell factory, based on the iAZ900 model (Zomorrodi and Maranas, 2010), was also developed through a collaboration within this project.

3.3 Metabolic modeling & engineering of the S. cerevisiae-EFE cell factory

The modeling of ethylene production in *S. cerevisiae* performed by Larsson et al. (2011) investigated several different conditions, including changing nitrogen source and altering the proline flux. In all their modeling they found that the respiratory activity was high, 5 $mole_{NADH}$ / $mole_{glucose}$. When limiting the respiration rate to 0.8 $mole_{NADH}$ / $mole_{glucose}$ the ethylene production was severely affected, decreasing from 0.78 $mole_{ethylene}$ / $mole_{glucose}$ to 0.02 $mole_{ethylene}$ / $mole_{glucose}$. They further showed that specifying the co-factor specificity of Gdh to NAD^+ increased the predicted ethylene levels with 7 %. Hence, it seems that the respiratory capacity is a limiting factor of ethylene production in *S. cerevisiae*. As mentioned in the first chapter oxygen restriction severely affected the ethylene formation *in vivo* already at relatively small changes in dissolved oxygen tension, this can then be linked to two reasons; the reaction of the enzyme itself for which oxygen is substrate, as well as to the requirement of oxygen for the increased usage in re-oxidation of NADH (**chapter 1**, **paper II**).

Different approaches can be used to meet this limitation. In **paper II** benzoic acid was added to increase the respiration rate and a positive effect was seen on the specific ethylene formation, however the biomass formation was severely reduced (as discussed in **chapter 2**). Further, *S. cerevisiae* lacks transhydrogenase activity (Bruinenberg et al., 1985) which means that an imbalance in NADH cannot be met by conversion to NADPH. Expression of the soluble transhydrogenase (sth) of *Azotobacter vinelandii* (Chung, 1970) (Fig. 15) did however not lead to an increase in ethylene formation (**unpublished data**). In **paper IV** expression of heterologous oxidases was investigated as a mean to meet the limitations. Two different oxidases were tested, the alternative oxidase (*AOX1*) of the fungi *Histoplasma capsulatum* and the water-forming NADH oxidase (*nox*) of the bacteria *Streptococcus pneumonia*, both of

which have been shown previously to increase respiration when expressed in *S. cerevisiae* (Johnson et al., 2003; Vemuri et al., 2007). The Aox1 has further been shown to localize to the mitochondria when expressed in *S. cerevisiae* (Vemuri et al., 2007) whereas the nox will affect the cytosolic NADH metabolism. Aox1 functions by using electrons from the electron transport chain to reduce molecular oxygen (Akhter et al., 2003), whereas nox catalyzes direct oxidation of NADH to NAD^+ using molecular oxygen (Auzat et al., 1999) (Fig. 15).

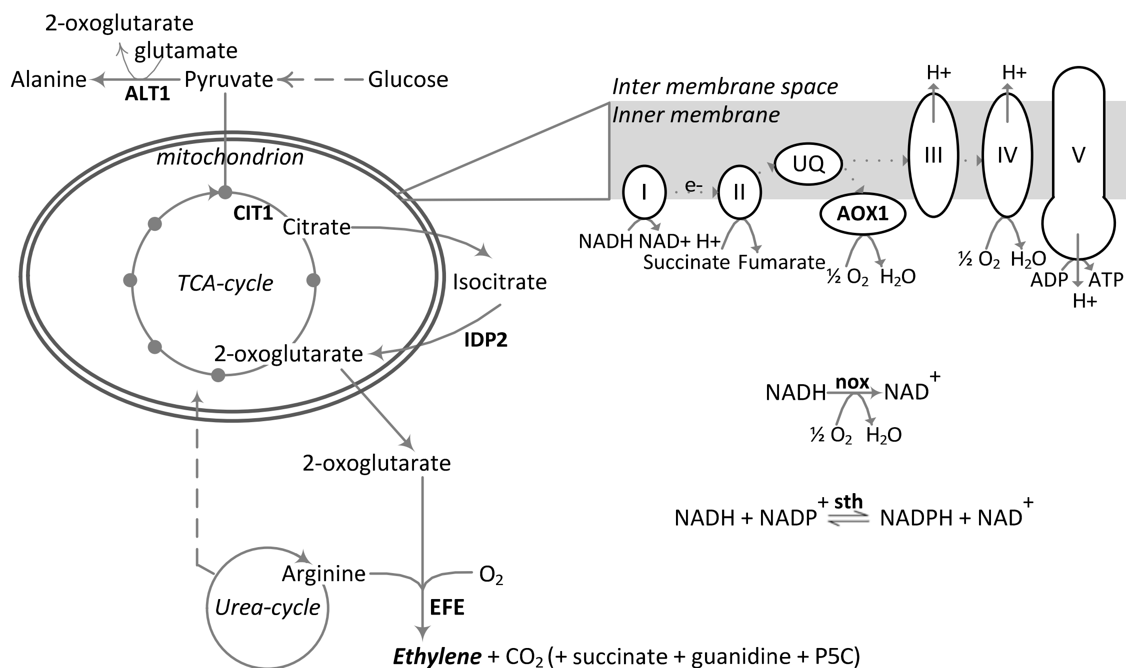


Figure 15. Metabolic engineering targets
in relation to the metabolism and respiratory chain of *S. cerevsiaie* as well as to the introduced EFE reaction. ALT1: Alanine transaminase, CIT1: Citrate synthase, IDP2: Isocitrate dehydrogenase, AOX1: Alternative oxidase of *Hisoplasma capsualtum*, nox: Water forming NADH oxidase of *Streptococcus penumoniae*, sth:Transhydrogenase of *Azotobacter vinelandii*

Both oxidases led to increased specific ethylene formation ($\mu g\ g_{DW}^{-1}\ h^{-1}$), however the effect of Aox1 on ethylene production was more pronounced (table 4). Further, both oxidase strains showed large increases in oxygen consumption indicating increased respiration rate. In the yeast the redox-metabolism is compartmentalized as the redox-couples cannot pass over the mitochondrial membrane, implying that the redox-state has to be balanced in the mitochondria and cytosol separately. During aerobic conditions mitochondrial NADH is re-oxidized in the

electron transport chain of *S. cerevisiae* whereas cytosolic NADH can be re-oxidized by the external NADH oxidases (Nde1p and Nde2p) (Luttik et al., 1998; Small and McAlister-Henn, 1998) or through the glycerol-3-phosphate shunt (Larsson et al., 1998), however both of these cytosolic systems channels the electrons of the NADH from the cytosol into the electron transport chain of the mitochondria (Rigoulet et al., 2004). As Aox1 functions by removing electrons from the electron transport chain, it might hence have an influence also on the cytosolic redox balance, which could be part of the explanation of the larger effect seen with Aox1. The expression of *AOX1* has further been shown to increase the expression of genes involved in the TCA cycle (Vemuri et al., 2007). Elevated levels of 2-oxoglutarate were also seen in our cultures, again linking increased 2-oxoglutarate levels to increased ethylene production as seen previously in **paper III**. nox on the other hand was shown to rather down-regulate expression of the Nde1p and Nde2p as well up-regulate NADH forming pathways, explaining the lesser effect of this enzyme on ethylene formation.

Table 4. Ethylene production in strains expressing heterologous oxidases

	Ethylene									
	Specific productivity μg g_{DW}^{-1} h^{-1}	±	**Productivity** μg L^{-1} h^{-1}	±	**Yield** μg $g^{-1}_{glucose}$	±	**Biomass** g L^{-1}	±	**qO2** mole O_2 h^{-1} g_{dw}^{-1}	±
REF	45,85	1,00	231,62	2,09	240,19	2,17	5,05	0,04	1,68	0,20
AOX	62,39	3,16	299,59	13,79	307,08	14,13	4,80	0,02	3,44	*
NOX	51,15	0,49	234,41	4,16	236,76	4,20	4,58	0,04	3,97	0,59

All strains contained an integrated copy of the EFE and the pYX212 plasmid either empty (REF) or with the Aox1 (AOX) or nox (NOX) gene. Cultivations performed as chemostats at D=0.1 h^{-1} and with 10g glucose L^{-1}.
± indicates min/max of duplicate steady states.
* only single measurement.

Larsson et al. (2011) concluded their paper with the suggestion that a more extensive genome scale metabolic model should be developed. This was performed in **paper IV**, where the iAZ900 model (Zomorrodi and Maranas, 2010) was extended with the complete ethylene reaction (reaction 3, page 11). FVA, where the fluxes of the ethylene model was compared those of the native yeast model, was then used to identify metabolic targets for increased ethylene formation. Growth rate was enforced to be at least 80% of the maximal rate and target ranking took ethylene and biomass formation rates as well as non-lethality and number of encoding genes into consideration. A list of the top 20 candidates was returned (table 5).

From this a selection of three candidates was made based on two criteria; the flux increase should be a minimum of 100% and the predicated increase in ethylene production rate should be at least 5 fold. The three selected target hence became; *CIT1* as a TCA cycle intermediate and mitochondrial target (Kim et al., 1986), *IDP2* as a representative of the cytoplasmic alternative to the TCA cycle (Haselbeck and Mcalister Henn, 1993), and finally *ALT1* as a non-TCA cycle intermediate with good supply of substrate (Garcia-Campusano et al., 2009) (Fig. 15). However, none of the overexpression strains showed any substantial increase in neither ethylene production nor in the extracellular metabolite pattern (data not shown).

From the above it becomes obvious that increasing the ethylene formation is difficult. Re-oxidation of NADH seems limiting, but can be met by co-expression of an alternative oxidase. However, attempts to increase the availability of the substrate 2-oxoglutarate through metabolic engineering did not give any results. As 2-oxoglutarate is linked to two central and heavily regulated metabolic networks; that of carbon and that of nitrogen, perhaps looking into regulation of these pathways could be an alternative strategy. It has been shown that for lysine production (a pathway also originating from 2-oxoglutarate) deletion of the pleiotropic negative regulator Mks1, resulted in both increased lysine production and a significantly increased intracellular 2-oxoglutarate concentrations without affecting growth rate (Quezada et al., 2013), this could hence be an interesting target.

Table 5. FVA results of metabolic model iAZ900 extended with the complete EFE reaction

Model1		
Name	Identifier	Formula
Ethylene exchange flux	EX_eth(e)	ethylene out <=>
Ethylene transporter	ethtex2	Ethylene in <=> ethylene out
Ethylene synthase	Ethylene_synthase_bact	3 2-oxoglutarate + arginine-L + 3 O_2 -> succinate + 7 CO_2 + 2 ethylene+ guanidine + 1-Pyrroline-5-carboxylate + 3 H_2O
Guanidine transporter	guadtex2	Guanidine in <=> guanidine out
Guanidine exchange flux		Guanidine out <=>

Name	Gene	Metabolic Process	Essential	Predicted fold change in expression[2]	Predicted fold increase in specific ethylene productivity
HOM6	YJR139C	Aspartate, 2-oxo and Arginine metabolism	yes	1,21	7,3
IDP2	YLR174W	TCA	yes	3,26	7,3
THR1	YHR025W	Aspartate, 2-oxo and Arginine metabolism	no	1,62	7,3
TPI1	YDR050C	TCA input flux	yes	1,05	7,3
TDH3 TDH1 TDH2	YGR192C YJL052W YJR009C	TCA input flux	yes	1,02	7,3
HOM2	YDR158W	Aspartate, 2-oxo and Arginine metabolism	yes	1,41	7,2
HOM3	YER052C	Aspartate, 2-oxo and Arginine metabolism	no	1,41	7,2
CIT1 CIT3	YNR001C YPR001W	TCA	yes	2,15	7,2
PYC2 PYC1	YBR218C YGL062W	TCA input flux	yes	2,25	6,9
RKI1	YOR095C	TCA input flux	yes	3,11	6,9
CDC1 (PYK1) PYK2	YAL038W YOR347C	TCA input flux	yes	1,10	6,7
MET10 MET5	YFR030W YJR137C	Aspartate, 2-oxo and Arginine metabolism	yes	1,57	6,7
THR4	YCR053W	Aspartate, 2-oxo and Arginine metabolism	no	1,43	6,7
IPP1	YBR011C	TCA input flux	yes	2,75	6,1
ALT2 (ALT1)	YDR111C	Aspartate consumption	yes	3,13	5,8
PMI40	YER003C	TCA input flux	no	1,20	5,7
PGK1	YCR012W	TCA input flux	yes	1,02	5,6
HIS1	YER055C	Utilization of asparate precursor	no	0,98	4,9
ADE1	YAR015W	Arginine precursor competition (from aspartate)	yes	0,74	3,6
ADE6	YGR061C	Arginine precursor competition (from aspartate)	yes	0,74	3,6

Target selection enforced a growth rate above 80 % of theoretical maximum.
A fold change below 1.0 means down-regulation, while one above 1.0 requires up-regulation.

CONCLUSIONS

In order to get a cell-factory to function at its optimal it is crucial to develop a deep knowledge about all the system parts as well as gaining a holistic understanding of the system. In this work I have aimed at extending the knowledge base of the EFE-*S. cerevisiae* system for ethylene production in order to enable evaluation of its suitability as a production entity.

Several issues have been addressed such as i) how does the introduction of EFE into *S. cerevisiae* effects the growth and metabolism of the host, ii) how can the metabolism of the host be improved for ethylene production, iii) increase the knowledge on the enzyme functionality, and if possible improve it in regards of ethylene production and iv) evaluate cultivation conditions in regards of improved ethylene production. Taken together the findings help evaluate the functionality if the system.

What has been learnt about the EFE, its structure and reaction process and which are the main challenges to be met regarding it?

Despite the fact that structural determination has proved difficult, several crucial structural features and amino acids have been identified using structure prediction tools and amino acid substitution. An unstructured loop has been identified as essential for ethylene formation, further a handful of amino acid residues have been found important either for substrate or iron binding (**paper I**). The proposed reaction mechanism of EFE is unusual and does not conform with the mechanism of related enzymes (**chapter 1**). To really untangle the reaction mechanism of this enzyme and thereby be able to truly relate it to the metabolism of the host I believe structure determination, preferably with and without substrate(s), is a must.

How suitable is S. cerevisiae as a host and how can its metabolism be further optimized in order to improve ethylene production?

When doing an inventory of the productivity of alternative cell factories for ethylene production tested until today *S. cerevisiae* stands out as one of the best host organisms. However, I have shown that its respiratory capacity limits ethylene formation (**paper II**). This can to some extent be relieved by the expression of heterologous oxidases (**paper IV**). Furthermore, a majority of the conditions which have given higher ethylene productivity have also shown increased levels of 2-oxoglutarate (**paper III** and **IV**). Increasing the net flux to this hence seems crucial. Rewiring of the fluxes through the central carbon or nitrogen metabolisms is however difficult, as shown in **paper III** and **IV**.

What have been learnt about optimal processing conditions for ethylene production via this cell factory and which challenges does the process of ethylene formation via the EFE stand in for?

Two crucial cultivation factors for ethylene formation have been identified during this study; 1) the oxygenation level has to be kept high to ensure good ethylene production, even relatively small decreases in oxygen availability will negatively affect the ethylene production (**paper II**). This effect can be connected to two separate issues; that oxygen is a substrate for the enzyme and that oxygen is required to meet an increased demand of NADH re-oxidation (**paper I1** and **IV**).

2) the nitrogen source used can have both a positive and a detrimental effect on the ethylene formation as glutamate increases levels whereas arginine reduced them. This is most likely connected to the metabolism of the compounds, as glutamate catabolism results in increased levels of the EFE substrate 2-oxoglutarate, whereas the catabolism of arginine consumes 2-oxoglutarate (**paper II and III**).

In an industrial setting both of these facts can be seen as problems. To cultivate the strain on glutamate is likely not relevant on an industrial scale, however more metabolic engineering strategies could be possible for increasing levels of 2-oxolgutarate. Further to maintain a high oxygenation level in large scale fermenters is expensive and difficult as stirring of large volumes often is non-homogenous and energy intensive.

Concluding remarks

My work has hence revealed several obstacles which must be overcome to make this system a viable biotechnical solution for ethylene production. It has also given hints about how to solve parts of them. In general I must conclude that there is a long, long way to go before an EFE-based ethylene cell-factory is a reality. It is implausible that the process will ever be as effective at a g/g basis as the bio-ethanol based production, however if one takes the entire production process into perspective, the outcome could be that the lower energy demand of the direct conversion could decrease the production costs enough to still make the technique economically competitive.

FUTURE DIRECTIONS

One of the main obstacles of the set-up used in this study, and which has become even more obvious during thus study, is the enzyme itself. One does ask oneself, did the original ethylene producers have that much to gain from producing ethylene that it would develop an enzyme specifically for ethylene production? Perhaps the main purpose of the enzyme really is to perform the non-ethylene forming reaction, and supply the cell with an alternative pathway for production of proline?

Interestingly it has been put forward that the flexibility in the reaction mechanism of the enzyme family as a whole is kept by the cell as a starting point for the development of new enzymes and enzymatic reactions (Hewitson et al., 2005). If this bears any truth is hard to say, however this work highlights that the low stability of the enzyme and the lack of steerability of its reactions are major issues which has to be addressed if the system is to ever have any future. This does rely heavily on the determination of the enzymes structure and true reaction mechanism. Learning from our results showing that 2-oxoglutarate is crucial for stability reasons as well as for the formation of crystal nucleuses (**unpublished data, chapter 1**), and combining this with the fact that crystallization of other enzymes containing a coordinating iron shows that it might be necessary to perform the crystallization under anaerobic conditions as to not oxidize the iron and disturb the enzyme structure (something we did not have the possibility to try) the basis for further crystallization experiments has been laid.

When it comes to NMR based structure determination, EFE is at the very maximum of what is achievable size wise with this technique today. This work was hence always a bit of a gamble, however the technique is ever evolving and within a not too far away future developments might have been accomplished which enables measurements of even larger enzymes at even lower enzyme concentrations. Instability issues of the enzyme during purification and measuring can partly be met by the addition of 2-oxoglutarate and arginine (as shown in **chapter 1**). The introduction of so-called solubilization tags (Zhou et al., 2001) could also be an alternative solution, however adding the tag does add to the size of the protein and the type of tag used has to be chosen with care.

The instability or misfolding of the EFE is likely a major issue also *in vivo* as the presence of inclusion bodies have been identified at least in *E. coli*. This needs to be met, though the difficulty of increasing the folding stability is well known there are methods to apply. We did show that altering the enzyme source to one phylogenetically closer to the host was not

beneficial for ethylene formation (**paper I**), likewise neither did the co-expression of the bacterial folding chaperones GroEL and GroES help ethylene formation via the bacterial *P. syringae* version (**unpublished data, chapter 1**). There are other ways to increase the folding stability of an enzyme, such as altering the charges of surface groups of the enzyme (Grimsley et al., 1999). This is however a major work, especially as long as the structure of the enzyme is unknown.

In **paper II** and **IV** I showed that the productivity of ethylene is intimately linked to respiration capacity and redox metabolism, hence more work can be done on this. Specifically, it has been suggested that altering the co-factor dependence of the glutamate dehydrogenase from $NADP^+$, which is usually used during growth on ammonia, to NAD^+ could improve the yields further (Larsson et al., 2011), this would hence be a good next step for metabolic engineering.

Paper II and **III** as well as the modeling results from **paper IV** showed the centrality of 2-oxoglutarate metabolism and provision for optimal ethylene formation. Many of the top targets of the metabolic modeling were enzymes involved in 2-oxoglutarate formation. Much more could be done with the information from the modeling and especially combining targets within the same pathway within one strain would be interesting for the further evaluation.

From our results one can question the suitability of the EFE production pathway and it would be of interest to evaluate other production option. However as discussed in this thesis the known alternative biological pathways (the plant version and the KMBA pathway) both have major drawbacks. Nevertheless there might be other alternatives, such as mimicking the bio-ethanol dehydration process, i.e. manufacturing a strain capable of direct enzymatic dehydration of formed ethanol. A patent for such a method was published in 2011 (Marliere, 2011) and could be worth looking into.

There are hence many alternative pathways to go in order to try and improve the direct biotechnological production of ethylene, but for now the ethanol based process remains by far the best bio-based alternative.

ACKNOWLEDGEMENT

On the surface research can seem to be an individual work, however accomplishing this PhD would not have been possible without the support from so many people.

First of all I would like to thank my supervisors, Christer Larsson and Joakim Norbeck, for taking me on as a PhD student and believing in me. Christer, your endless optimism has been a source of inspiration during this sometimes very unruly project. Joakim, you are indeed very right in your analysis that finishing a PhD is as much about handling frustration as it is about science sometimes. Thank you! to the both of you for sharing your deep scientific knowledge with me and a lot of laughter.

I would also like to thank Anders Pedersen and Göran Karlsson at the Swedish NMR center at Gothenburg University. Especially Anders for your unending patience while teaching me the ins and outs of protein production, purification and function analysis.

An acknowledgement is also due for Stéphanie Heux, Gillles Vieira and Ludovic Cottret at LISBP – INRA Toulouse for interesting scientific discussions regarding the metabolic modeling and optimization of ethylene production.

During my time as a PhD student I have also had the pleasure of supervising two eminent master students; Paul Quehl and Karl Persson. You have both contributed immensely to this research and I am sure both of you will have great futures, whatever you set your minds to.

A large thank you further goes to the personnel who make sure everything works as smooth as possible; Erica Dahlin, Helena Janveden, Martina Butorac, Ximena Rozo Sevilla, Suwanee Jansa-Ard, Marie Nordqvist, Emma Ribbenhed, Jenny Nilsson, Julia Karlsson, Andreas Hellström and Malin Nordvall.

To everybody in the Sys2Bio and Indbio groups. Payam, sharing the small office with you has been a pleasure. Bouke, cheers for all the sauna nights, dinners, chats and encouraging words. To Christoph, Sakda, Kaisa, Rahul, Lina, Emma, Petri, Eugenio, Ana, Natapol, Kanokarn & Pramote thank you for making this time about so much more than work and to all the rest of you in the groups for making this such an enjoyable place to be. Also to all of my friends outside of work, you have kept me sane and given me well needed breaks from my scientific struggles.

Finally, to my family, I am at a loss for words as how to thank you enough. Mom and dad, without your unconditional love and belief in me I would not have had the confidence to even start this journey. Sis, thank you for making me laugh when I cry and for making me laugh until I cry. And Perka (Per-Erik), without you I would not have made it to the end of this! Thank you for your endless patience, understanding and support, I love you.

A tremendous feeling of peace came over him.

He knew that at last, for once and for ever, it was now all, finally, over.

Douglas Adams (Mostly Harmless)

REFERENCES

Adams, D. O., Yang, S. F., 1979. Ethylene Biosynthesis - Identification of 1-Aminocyclopropane-1-Carboxylic Acid as an Intermediate in the Conversion of Methionine to Ethylene. P Natl Acad Sci USA. 76, 170-174.

Aguayo, A. T., Gayubo, A. G., Atutxa, A., Olazar, M., Bilbao, J., 2002. Catalyst deactivation by coke in the transformation of aqueous ethanol into hydrocarbons. Kinetic modeling and acidity deterioration of the catalyst. Ind Eng Chem Res. 41, 4216-4224.

Akhter, S., McDade, H. C., Gorlach, J. M., Heinrich, G., Cox, G. M., Perfect, J. R., 2003. Role of alternative oxidase gene in pathogenesis of *Cryptococcus neoformans*. Infect Immun. 71, 5794-5802.

Albers, E., 2000. Nitrogen and redox metabolism in *Saccharomyces cerevisiae*. Chalmers tekniska högsk., Göteborg.

Albers, E., Gustafsson, L., Niklasson, C., Liden, G., 1998. Distribution of C-14-labelled carbon from glucose and glutamate during anaerobic growth of *Saccharomyces cerevisiae*. Microbiol-Uk. 144, 1683-1690.

Albers, E., Larsson, C., Liden, G., Niklasson, C., Gustafsson, L., 1996. Influence of the nitrogen source on *Saccharomyces cerevisiae* anaerobic growth and product formation. Appl Environ Microb. 62, 3187-3195.

Araki, S., Matsuoka, M., Tanaka, M., Yoshida, K., Shinmyo, A., Ogawa, T., 2001. Expression of an ethylene-forming enzyme from Pseudomonas syringae under the control of alcohol dehydrogenase gene promoter in tobacco roots. Biotechnol Lett. 23, 433-436.

Argueso, C. T., Hansen, M., Kieber, J. J., 2007. Regulation of ethylene biosynthesis. J Plant Growth Regul. 26, 92-105.

Asadollahi, M. A., Maury, J., Patil, K. R., Schalk, M., Clark, A., Nielsen, J., 2009. Enhancing sesquiterpene production in *Saccharomyces cerevisiae* through in silico driven metabolic engineering. Metab Eng. 11, 328-334.

Ask, M., Olofsson, K., Di Felice, T., Ruohonen, L., Penttila, M., Liden, G., Olsson, L., 2012. Challenges in enzymatic hydrolysis and fermentation of pretreated Arundo donax revealed by a comparison between SHF and SSF. Process Biochem. 47, 1452-1459.

Attfield, P. V., Bell, P. J. L., 2006. Use of population genetics to derive nonrecombinant *Saccharomyces cerevisiae* strains that grow using xylose as a sole carbon source. Fems Yeast Res. 6, 862-868.

Auzat, I., Chapuy-Regaud, S., Le Bras, G., Dos Santos, D., Ogunniyi, A. D., Le Thomas, I., Garel, J. R., Paton, J. C., Trombe, M. C., 1999. The NADH oxidase of *Streptococcus pneumoniae*: its involvement in competence and virulence. Mol Microbiol. 34, 1018-1028.

Avendano, A., Deluna, A., Olivera, H., Valenzuela, L., Gonzalez, A., 1997. GDH3 encodes a glutamate dehydrogenase isozyme, a previously unrecognized route for glutamate biosynthesis in *Saccharomyces cerevisiae*. J Bacteriol. 179, 5594-5597.

Bailey, J. E., 1991. Toward a Science of Metabolic Engineering. Science. 252, 1668-1675.

Baker, D., Sali, A., 2001. Protein structure prediction and structural genomics. Science. 294, 93-96.

Bame, D., Fehler, M., 1986. Observations of Long Period Earthquakes Accompanying Hydraulic Fracturing. Geophys Res Lett. 13, 149-152.

Bar-Even, A., Flamholz, A., Noor, E., Milo, R., 2012. Rethinking glycolysis: on the biochemical logic of metabolic pathways. Nat Chem Biol. 8, 509-517.

Barnett, J. A., 1997. Sugar utilization by *Saccharomyces cerevisiae*. In: Zimmermann, F. K., Entian, K. D., Eds.), Yeast sugar metabolism; biochemistry, genetics, biotechnology and applications. Technomic Publishing Company, Lancaster, PA, pp. 35-44.

Bassi, P. K., Eastwell, K. C., Akalehiywot, T., Spencer, M. S., 1981. Methods for Quantitative-Determination of Ethylene in Aqueous-Solutions for Biological Studies. Plant Cell Environ. 4, 271-274.

Basso, L. C., Basso, T. O., Rocha, S. N., 2011. Ethanol production in Brazil: the industrial process and its impact on yeast fermentation. In: Bernardes, M. A. S., (Ed.), Biofuel production - recent developments and prospects. InTech, pp. 85-100.

Bi, J. D., Guo, X. W., Liu, M., Wang, X. S., 2010. High effective dehydration of bio-ethanol into ethylene over nanoscale HZSM-5 zeolite catalysts. Catal Today. 149, 143-147.

Biale, J. B., 1940. Effect of emanations from;several species of fungi on respiration and color development of citrus fruits. Science. 91, 458-459.

Binder, B. M., 2008. The ethylene receptors: Complex perception for a simple gas. Plant Sci. 175, 8-17.

Boer, V. M., Crutchfield, C. A., Bradley, P. H., Botstein, D., Rabinowitz, J. D., 2010. Growth-limiting Intracellular Metabolites in Yeast Growing under Diverse Nutrient Limitations. Mol Biol Cell. 21, 198-211.

Brandriss, M. C., Magasanik, B., 1980. Proline - an Essential Intermediate in Arginine Degradation in Saccharomyces-Cerevisiae. J Bacteriol. 143, 1403-1410.

Brandriss, M. C., Magasanik, B., 1981. Subcellular Compartmentation in Control of Converging Pathways for Proline and Arginine Metabolism in Saccharomyces-Cerevisiae. J Bacteriol. 145, 1359-1364.

Bro, C., Regenberg, B., Forster, J., Nielsen, J., 2006. In silico aided metabolic engineering of Saccharomyces cerevisiae for improved bioethanol production. Metab Eng. 8, 102-111.

Bruinenberg, P. M., Jonker, R., Vandijken, J. P., Scheffers, W. A., 1985. Utilization of Formate as an Additional Energy-Source by Glucose-Limited Chemostat Cultures of *Candida utilis* Cbs-621 and *Saccharomyces cerevisiae* Cbs-8066 - Evidence for the Absence of Transhydrogenase Activity in Yeasts. Arch Microbiol. 142**,** 302-306.

Buschke, N., Schafer, R., Becker, J., Wittmann, C., 2013. Metabolic engineering of industrial platform microorganisms for biorefinery applications - Optimization of substrate spectrum and process robustness by rational and evolutive strategies. Bioresource Technol. 135**,** 544-554.

Carlson, M., 1999. Glucose repression in yeast. Curr Opin Microbiol. 2**,** 202-207.

Cavani, F., Ballarini, N., Cericola, A., 2007. Oxidative dehydrogenation of ethane and propane: How far from commercial implementation? Catal Today. 127**,** 113-131.

Chen, X., Liang, Y., Hua, J., Tao, L., Qin, W. S., Chen, S. F., 2010. Overexpression of bacterial ethylene-forming enzyme gene in Trichoderma reesei enhanced the production of ethylene. International Journal of Biological Sciences. 6**,** 96-106.

Chung, A. E., 1970. Pyridine Nucleotide Transhydrogenase from *Azotobacter vinelandii*. J Bacteriol. 102**,** 438-&.

Cogoni, C., Valenzuela, L., Gonzalezhalphen, D., Olivera, H., Macino, G., Ballario, P., Gonzalez, A., 1995. Saccharomyces-Cerevisiae Has a Single Glutamate Synthase Gene Coding for a Plant-Like High-Molecular-Weight Polypeptide. J Bacteriol. 177**,** 792-798.

Cooper, T., 1982. Nitrogen metabolism in *Saccharomyces cerevisiae*. In: Strathern, J. N., Jones, E. W., Broach, J. R., Eds.), The Molecular Biology of the Yeast *Saccharomyces cerevisiae*: Metabolism and Gene Expression. vol. 1. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY., pp. 39-99.

Cooper, T. G., 2002. Transmitting the signal of excess nitrogen in *Saccharomyces cerevisiae* from the Tor proteins to the GATA factors: connecting the dots. Fems Microbiol Rev. 26**,** 223-238.

Cortassa, S., Aon, M. A., 1998. The onset of fermentative metabolism in continuous cultures depends on the catabolite repression properties of Saccharomyces cerevisiae. Enzyme Microb Tech. 22**,** 705-712.

Crabtree, H. G., 1929. Observations on the carbohydrate metabolism of tumours. Biochem J. 23**,** 536-545.

Crepin, L., Nidelet, T., Sanchez, I., Dequin, S., Camarasa, C., 2012. Sequential Use of Nitrogen Compounds by Saccharomyces cerevisiae during Wine Fermentation: a Model Based on Kinetic and Regulation Characteristics of Nitrogen Permeases. Appl Environ Microb. 78**,** 8102-8111.

Crick, F., 1970. Central Dogma of Molecular Biology. Nature. 227**,** 561-&.

Daran-Lapujade, P., Daran, J. M., Kotter, P., Petit, T., Piper, M. D. W., Pronk, J. T., 2003. Comparative genotyping of the Saccharomyces cerevisiae laboratory strains S288C and CEN.PK113-7D using oligonucleotide microarrays. Fems Yeast Res. 4**,** 259-269.

Daum, G., Tuller, G., Nemec, T., Hrastnik, C., Balliano, G., Cattel, L., Milla, P., Rocco, F., Conzelmann, A., Vionnet, C., Kelly, D. E., Kelly, S., Schweizer, E., Schuller, H. J., Hojad, U., Greiner, E., Finger, K., 1999. Systematic analysis of yeast strains with possible defects in lipid metabolism. Yeast. 15**,** 601-614.

De Deken, R. H., 1966. Crabtree Effect - a Regulatory System in Yeast. J Gen Microbiol. 44**,** 149-156.

de Jong, L., Kemp, A., 1984. Stoicheiometry and Kinetics of the Prolyl 4-Hydroxylase Partial Reaction. Biochim Biophys Acta. 787**,** 105-111.

DeLuna, A., Avendano, A., Riego, L., Gonzalez, A., 2001. NADP-glutamate dehydrogenase isoenzymes of *Saccharomyces cerevisiae* - Purification, kinetic properties, and physiological roles. J Biol Chem. 276**,** 43775-43783.

Doucette, C. D., Schwab, D. J., Wingreen, N. S., Rabinowitz, J. D., 2011. alpha-ketoglutarate coordinates carbon and nitrogen utilization via enzyme I inhibition. Nat Chem Biol. 7**,** 894-901.

Duarte, N. C., Herrgård, M. J., Palsson, B. O., 2004. Reconstruction and validation of *Saccharomyces cerevisiae* iND750, a fully compartmentalized genome-scale metabolic model. Genome Res. 14**,** 1298-1309.

Duncan, H. M., Mackler, B., 1966. Electron Transport Systems of Yeast .3. Preparation and Properties of Cytochrome Oxidase. J Biol Chem. 241**,** 1694-&.

Eckert, C., Xu, W., Xiong, W., Lynch, S., Ungerer, J., Tao, L., Gill, R., Maness, P. C., Yu, J. P., 2014. Ethylene-forming enzyme and bioethylene production. Biotechnol Biofuels. 7.

Elbing, K., Larsson, C., Bill, R. M., Albers, E., Snoep, J. L., Boles, E., Hohmann, S., Gustafsson, L., 2004. Role of hexose transport in control of glycolytic flux in *Saccharomyces cerevisiae*. Appl Environ Microb. 70**,** 5323-5330.

European Commssion, Innovating for Sustainable Growth: A Bioeconomy for Europe, COM(2012) 60 final. 2012.

Fan, D., Dai, D. J., Wu, H. S., 2013. Ethylene Formation by Catalytic Dehydration of Ethanol with Industrial Considerations. Materials. 6**,** 101-115.

Fiechter, A., Fuhrmann, G. F., Käppeli, O., 1981. Regulation of Glucose Metabolism in Growing Yeast Cells. Advances in Microbial Physiology. 22**,** 123-183.

Fraenkel, D. G., 1982. Carbohydrate metabolism. In: Strathern, J. N., Jones, E. W., Broach, J. R., Eds.), The molecular biology of the Yeast *Saccharmyces cerevisiae*: Metabolism and Gene Expression. vol. 1. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY, pp. 1-38.

Fraenkel, D. G., 2011. Yeast intermediary metabolism. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Frick, O., Wittmann, C., 2005. Characterization of the metabolic shift between oxidative and fermentative growth in *Saccharomyces cerevisiae* by comparative C-13 flux analysis. Microb Cell Fact. 4**,** 30.

Fukuda, H., Ogawa, T., Tanase, S., 1993. Ethylene Production by Microorganisms. Adv Microb Physiol. 35**,** 275-306.

Fukuda, H., Ogawa, T., Tazaki, M., Nagahama, K., Fujii, T., Tanase, S., Morino, Y., 1992. 2 Reactions Are Simultaneously Catalyzed by a Single Enzyme - the Arginine-Dependent Simultaneous Formation of 2 Products, Ethylene and Succinate, from 2-Oxoglutarate by an Enzyme from *Pseudomonas syringae*. Biochem Bioph Res Co. 188**,** 483-489.

Förster, J., Famili, I., Fu, P., Palsson, B. O., Nielsen, J., 2003. Genome-scale reconstruction of the *Saccharomyces cerevisiae* metabolic network. Genome Res. 13**,** 244-253.

Galbe, M., Sassner, P., Wingren, A., Zacchi, G., 2007. Process engineering economics of bioethanol production. Adv Biochem Eng Biot. 108**,** 303-327.

Garcia-Campusano, F., Anaya, V. H., Robledo-Arratia, L., Quezada, H., Hernandez, H., Riego, L., Gonzalez, A., 2009. ALT1-encoded alanine aminotransferase plays a central role in the metabolism of alanine in *Saccharomyces cerevisiae*. Can J Microbiol. 55**,** 368-374.

Ghanta, M., Fahey, D., Subramaniam, B., 2013. Environmental impacts of ethylene production from diverse feedstocks and energy sources. Appl Petrochem Res. 1-13.

Godard, P., Urrestarazu, A., Vissers, S., Kontos, K., Bontempi, G., van Helden, J., Andre, B., 2007. Effect of 21 different nitrogen sources on global gene expression in the yeast *Saccharomyces cerevisiae*. Mol Cell Biol. 27**,** 3065-3086.

Goffeau, A., Barrell, B. G., Bussey, H., Davis, R. W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J. D., Jacq, C., Johnston, M., Louis, E. J., Mewes, H. W., Murakami, Y., Philippsen, P., Tettelin, H., Oliver, S. G., 1996. Life with 6000 genes. Science. 274**,** 546, 563-567.

Grimsley, G. R., Shaw, K. L., Fee, L. R., Alston, R. W., Huyghues-Despointes, B. M. P., Thurlkill, R. L., Scholtz, J. M., Pace, C. N., 1999. Increasing protein stability by altering long-range coulombic interactions. Protein Sci. 8**,** 1843-1849.

Gu, H. Q., Zhang, J., Bao, J., 2014. Inhibitor analysis and adaptive evolution of Saccharomyces cerevisiae for simultaneous saccharification and ethanol fermentation from industrial waste corncob residues. Bioresource Technol. 157**,** 6-13.

Gurgul, J., Zimowska, M., Mucha, D., Socha, R. P., Matachowski, L., 2011. The influence of surface composition of Ag3PW12O40 and Ag3PMo12O40 salts on their catalytic activity in dehydration of ethanol. J Mol Catal a-Chem. 351**,** 1-10.

Haselbeck, R. J., Mcalister Henn, L., 1993. Function and Expression of Yeast Mitochondrial NAD-Specific and NADP-Specific Isocitrate Dehydrogenases. J Biol Chem. 268, 12116-12122.

Hausinger, R. P., 2004. Fe(II)/alpha-ketoglutarate-dependent hydroxylases and related enzymes. Crit Rev Biochem Mol. 39, 21-68.

Hazan, R., Levine, A., Abeliovich, H., 2004. Benzoic acid, a weak organic acid food preservative, exerts specific effects on intracellular membrane trafficking pathways in *Saccharomyces cerevisiae*. Appl Environ Microb. 70, 4449-4457.

Hazelwood, L. A., Daran, J. M., van Maris, A. J. A., Pronk, J. T., Dickinson, J. R., 2008. The ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae metabolism* Appl Environ Microb. 74, 2259-2266.

Herrgård, M. J., Lee, B. S., Portnoy, V., Palsson, B. O., 2006. Integrated analysis of regulatory and metabolic networks reveals novel regulatory mechanisms in *Saccharomyces cerevisiae*. Genome Res. 16, 627-635.

Herrgård, M. J., Swainston, N., Dobson, P., Dunn, W. B., Arga, K. Y., Arvas, M., Bluthgen, N., Borger, S., Costenoble, R., Heinemann, M., Hucka, M., Le Novere, N., Li, P., Liebermeister, W., Mo, M. L., Oliveira, A. P., Petranovic, D., Pettifer, S., Simeonidis, E., Smallbone, K., Spasic, I., Weichart, D., Brent, R., Broomhead, D. S., Westerhoff, H. V., Kirdar, B., Penttila, M., Klipp, E., Palsson, B. O., Sauer, U., Oliver, S. G., Mendes, P., Nielsen, J., Kell, D. B., 2008. A consensus yeast metabolic network reconstruction obtained from a community approach to systems biology. Nat Biotechnol. 26, 1155-1160.

Hewitson, K. S., Granatino, N., Welford, R. W. D., McDonough, M. A., Schofield, C. J., 2005. Oxidation by 2-oxoglutarate oxygenases: non-haem iron systems in catalysis and signalling. Philos T Roy Soc A. 363, 807-828.

Heyland, J., Fu, J. A., Blank, L. M., 2009. Correlation between TCA cycle flux and glucose uptake rate during respiro-fermentative growth of *Saccharomyces cerevisiae*. Microbiol-Sgm. 155, 3827-3837.

Hofman-Bang, J., 1999. Nitrogen catabolite repression in *Saccharomyces cerevisiae*. Mol Biotechnol. 12, 35-73.

Holloway, M. D., Rudd, O., 2013. Production Development. Fracking : The Operations and Environmental Consequences of Hydraulic Fracturing. John Wiley & Sons, Somerset, NJ, pp. 5-10.

Holmes, A. R., Mcnaughton, G. S., More, R. D., Shepherd, M. G., 1991. Ammonium Assimilation by Candida-Albicans and Other Yeasts - a N-13 Isotope Study. Can J Microbiol. 37, 226-232.

Holyoak, C. D., Bracey, D., Piper, P. W., Kuchler, K., Coote, P. J., 1999. The *Saccharomyces cerevisiae* weak-acid-inducible ABC transporter pdr12 transports fluorescein and preservative anions from the cytosol by an energy-dependent mechanism. J Bacteriol. 181, 4644-4652.

Hong, K. K., Nielsen, J., 2012. Metabolic engineering of *Saccharomyces cerevisiae*: a key cell factory platform for future biorefineries. Cell Mol Life Sci. 69, 2671-2690.

Ishihara, K., Matsuoka, M., Inoue, Y., Tanase, S., Ogawa, T., Fukuda, H., 1995. Overexpression and *in Vitro* Reconstitution of the Ethylene Forming Enzyme from *Pseudomonas syringae*. J Ferment Bioeng. 79, 205-211.

Ishihara, K., Matsuoka, M., Ogawa, T., Fukuda, H., 1996. Ethylene production using a broad-host-range plasmid in *Pseudomonas syringae* and *Pseudomonas putida*. J Ferment Bioeng. 82, 509-511.

Jauniaux, J. C., Urrestarazu, L. A., Wiame, J. M., 1978. Arginine Metabolism in *Saccharomyces cerevisiae* - Subcellular Localization of Enzymes. J Bacteriol. 133, 1096-1107.

Johnson, C. H., Prigge, J. T., Warren, A. D., McEwen, J. E., 2003. Characterization of an alternative oxidase activity of *Histoplasma capsulatum*. Yeast. 20, 381-388.

Jones, E. W., Fink, G. R., 1982. Regulation of amino acid and nucleotide biosynthesis in yeast. In: Strathern, J. N. J., E. W., Broach, J. R., Eds.), The Molecular Biology of the Yeast *Saccharomyces cerevisiae*: Metabolism and Gene Expression. Cold Sping Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 181-299.

Kawachi, S., Arao, T., Suzuki, Y., Tamura, K., 2010. Effects of compressed unsaturated hydrocarbon gases on yeast growth. High-Pressure Bioscience and Biotechnology. 1189, 121-126.

Kelley, L. A., Sternberg, M. J. E., 2009. Protein structure prediction on the Web: a case study using the Phyre server. Nat Protoc. 4, 363-371.

Key, T. E., 1945. The history of sugical anesthesia. Wood Library - Museum of Anesthesiology, Park Ridge, IL.

Kim, K. S., Rosenkrantz, M. S., Guarente, L., 1986. *Saccharomyces cerevisiae* Contains 2 Functional Citrate Synthase Genes. Mol Cell Biol. 6, 1936-1942.

Koottungal, L., 2012. International survey of ethylene from steam crackers - 2012. Oil Gas J. 110, 85-93.

Koppram, R., Tomas-Pejo, E., Xiros, C., Olsson, L., 2014. Lignocellulosic ethanol production at high-gravity: challenges and perspectives. Trends Biotechnol. 32, 46-53.

Kuepfer, L., Sauer, U., Blank, L. M., 2005. Metabolic functions of duplicate genes in Saccharomyces cerevisiae. Genome Res. 15, 1421-1430.

Käppeli, O., 1986. Regulation of Carbon Metabolism in *Saccharomyces cerevisiae* and Related Yeasts. Advances in Microbial Physiology. 28, 181-209.

Larsson, C., Pahlman, I. L., Ansell, R., Rigoulet, M., Adler, L., Gustafsson, L., 1998. The importance of the glycerol 3-phosphate shuttle during aerobic growth of Saccharomyces cerevisiae. Yeast. 14, 347-357.

Larsson, C., Snoep, J. L., Norbeck, J., Albers, E., 2011. Flux balance analysis for ethylene formation in genetically engineered *Saccharomyces cerevisiae*. Iet Syst Biol. 5**,** 245-251.

Lewis, M. J., Rainbow, C., 1963. Transamination and the liberation of 2-oxoglutarate by yeast. J I Brewing. 69**,** 39-45.

Lin, Z. F., Zhong, S. L., Grierson, D., 2009. Recent advances in ethylene research. J Exp Bot. 60**,** 3311-3336.

Livingstone, H. M., 1955. 3 Decades of Ethylene-Oxygen Anesthesia. Anesthesiology. 16**,** 224-229.

Ljungdahl, P. O., Daignan-Fornier, B., 2012. Regulation of Amino Acid, Nucleotide, and Phosphate Metabolism in *Saccharomyces cerevisiae*. Genetics. 190**,** 885-929.

Lu, B. W., Yu, B., Li, N., 1999. Expression of a novel ethylene-producing bifunctional fusion enzyme in yeast. Bot Bull Acad Sinica. 40**,** 107-114.

Luttik, M. A. H., Overkamp, K. M., Kotter, P., de Vries, S., van Dijken, J. P., Pronk, J. T., 1998. The *Saccharomyces cerevisiae* NDE1 and NDE2 genes encode separate mitochondrial NADH dehydrogenases catalyzing the oxidation of cytosolic NADH. J Biol Chem. 273**,** 24529-24534.

Magasanik, B., Kaiser, C. A., 2002. Nitrogen regulation in *Saccharomyces cerevisiae*. Gene. 290**,** 1-18.

Mahadevan, R., Schilling, C. H., 2003. The effects of alternate optimal solutions in constraint-based genome-scale metabolic models. Metab Eng. 5**,** 264-276.

Mann, P., Walsh, P. R., Mackey, P. J., Jaising, V., Hwang, H. H., Chen, J. C., Maheshwari, M., Dan, C. A., Petrochemicals: Preparing for a supercycle. Morgan Stanley Blue Paper, 2010.

Marliere, P., Method for producing an alkene comprising the step of converting an alcohol by an enzymatic dehydration step. Vol. EP 2336341 A1, 2011.

Matar, S., Lewis, F., 2001a. Crude oil processing and production of hydrocarbon intermediates. Chemistry of Petrochemical processes. Gulf Prefssional Publishing, Woburn, MA, pp. 49-110.

Matar, S., Lewis, F., 2001b. Primary raw materials for petrochemicals. Chemistry of Petrochemical processing. Gulf Professional Publishing, Woburn, MA, pp. 1-28.

McCoy, M., Reisch, M., Tullo, A. H., Short, P. L., Tremblay, J.-F., Storck, W. J., 2006. Production: Growth is the norm. Chem Eng News. 84**,** 59-68.

McGovern, P. E., Glusker, D. L., Exner, L. J., Voigt, M. M., 1996. Neolithic resinated wine. Nature. 381**,** 480-481.

McGovern, P. E., Zhang, J. H., Tang, J. G., Zhang, Z. Q., Hall, G. R., Moreau, R. A., Nunez, A., Butrym, E. D., Richards, M. P., Wang, C. S., Cheng, G. S., Zhao, Z. J., Wang, C.

S., 2004. Fermented beverages of pre- and proto-historic China. P Natl Acad Sci USA. 101, 17593-17598.

McManus, M. T., 2012. Annual Plant Reviews : Plant Hormone Ethylene. John Wiley & Sons, Hoboken, NJ, USA.

Middelhoven, W. J., 1964. Pathway of Arginine Breakdown in *Saccharomyces cerevisiae*. Biochim Biophys Acta. 93, 650-&.

Miller, E. V., Winston, J. R., Fisher, D. F., 1940. Production of epinasty by emanations from normal and decaying citrus fruits and from *Penicillium digitatum*. J Agric Res. 60, 0269-0277.

Mitchell, A. P., Magasanik, B., 1983. Purification and Properties of Glutamine-Synthetase from *Saccharomyces cerevisiae*. J Biol Chem. 258, 119-124.

Mo, M. L., Palsson, B. O., Herrgard, M. J., 2009. Connecting extracellular metabolomic measurements to intracellular flux states in yeast. Bmc Syst Biol. 3.

Nagahama, K., Ogawa, T., Fujii, T., Fukuda, H., 1992. Classification of Ethylene-Producing Bacteria in Terms of Biosynthetic Pathways to Ethylene. J Ferment Bioeng. 73, 1-5.

Nagahama, K., Ogawa, T., Fujii, T., Tazaki, M., Goto, M., Fukuda, H., 1991a. L-Arginine Is Essential for the Formation *In vitro* of Ethylene by an Extract of *Pseudomonas syringae*. J Gen Microbiol. 137, 1641-1646.

Nagahama, K., Ogawa, T., Fujii, T., Tazaki, M., Tanase, S., Morino, Y., Fukuda, H., 1991b. Purification and Properties of an Ethylene Forming Enzyme from *Pseudomonas syringae* Pv Phaseolicola Pk2. J Gen Microbiol. 137, 2281-2286.

Nagahama, K., Yoshino, K., Matsuoka, M., Tanase, S., Ogawa, T., Fukuda, H., 1998. Site-directed mutagenesis of histidine residues in the ethylene forming enzyme from *Pseudomonas syringae*. J Ferment Bioeng. 85, 255-258.

Nagasu, T., Hall, B. D., 1985. Nucleotide-Sequence of the Gdh Gene Coding for the Nadp-Specific Glutamate-Dehydrogenase of *Saccharomyces cerevisiae*. Gene. 37, 247-253.

Nickerson, W. J., 1948. Ethylene as a Metabolic Product of the Pathogenic Fungus, Blastomyces-Dermatitidis. Arch Biochem. 17, 225-233.

Niederberger, P., Miozzari, G., Hutter, R., 1981. Biological Role of the General Control of Amino Acid Biosynthesis in *Saccharomyces cerevisiae*. Mol Cell Biol. 1, 584-593.

Nielsen, J., 1995. Physiological engineering aspects of *Penicillium chrysogenum*. Polyteknisk forlag, Denmark.

Nissen, T. L., Schulze, U., Nielsen, J., Villadsen, J., 1997. Flux distributions in anaerobic, glucose-limited continuous cultures of *Saccharomyces cerevisiae*. Microbiol-Uk. 143, 203-218.

Nookaew, I., Jewett, M. C., Meechai, A., Thammarongtham, C., Laoteng, K., Cheevadhanarak, S., Nielsen, J., Bhumiratana, S., 2008. The genome-scale metabolic

model iIN800 of *Saccharomyces cerevisiae* and its validation: a scaffold to query lipid metabolism. Bmc Syst Biol. 2.

Noor, E., Eden, E., Milo, R., Alon, U., 2010. Central Carbon Metabolism as a Minimal Biochemical Walk between Precursors for Biomass and Energy. Mol Cell. 39**,** 809-820.

Ogawa, T., Takahashi, M., Fujii, T., Tazaki, M., Fukuda, H., 1990. The Role of Nadh-Fe(Iii)Edta Oxidoreductase in Ethylene Formation from 2-Keto-4-Methylthiobutyrate. J Ferment Bioeng. 69**,** 287-291.

Osborn, S. G., Vengosh, A., Warner, N. R., Jackson, R. B., 2011. Methane contamination of drinking water accompanying gas-well drilling and hydraulic fracturing. P Natl Acad Sci USA. 108**,** 8172-8176.

OSTP, National Bioeconomy Blueprint. 2012.

Otero, J. M., Nielsen, J., 2010. Industrial Systems Biology. Biotechnol Bioeng. 105**,** 439-460.

Otterstedt, K., Larsson, C., Bill, R. M., Ståhlberg, A., Boles, E., Hohmann, S., Gustafsson, L., 2004. Switching the mode of metabolism in the yeast *Saccharomyces cerevisiae*. Embo Rep. 5**,** 532-537.

Oxford Dictionaries. Vol. 2014. Oxford Univeristy Press, http://www.oxforddictionaries.com, 2014.

Palmieri, L., Lasorsa, F. M., Vozza, A., Agrimi, G., Fiermonte, G., Runswick, M. J., Walker, J. E., Palmieri, F., 2000. Identification and functions of new transporters in yeast mitochondria. Bba-Bioenergetics. 1459**,** 363-369.

Pirkov, I., Albers, E., Norbeck, J., Larsson, C., 2008. Ethylene production by metabolic engineering of the yeast *Saccharomyces cerevisiae*. Metab Eng. 10**,** 276-280.

Postma, E., Verduyn, C., Scheffers, W. A., Vandijken, J. P., 1989. Enzymic Analysis of the Crabtree Effect in Glucose Limited Chemostat Cultures of *Saccharomyces cerevisiae*. Appl Environ Microb. 55**,** 468-477.

Pronk, J. T., Steensma, H. Y., van Dijken, J. P., 1996. Pyruvate metabolism in *Saccharomyces cerevisiae*. Yeast. 12**,** 1607-1633.

Quezada, H., Marin-Hernandez, A., Arreguin-Espinosa, R., Rumjanek, F. D., Moreno-Sanchez, R., Saavedra, E., 2013. The 2-oxoglutarate supply exerts significant control on the lysine synthesis flux in Saccharomyces cerevisiae. Febs J. 280**,** 5737-5749.

Regenberg, B., Grotkjaer, T., Winther, O., Fausboll, A., Åkesson, M., Bro, C., Hansen, L. K., Brunak, S., Nielsen, J., 2006. Growth-rate regulated genes have profound impact on interpretation of transcriptome profiling in *Saccharomyces cerevisiae*. Genome Biol. 7**,** R107.

Reifenberger, E., Boles, E., Ciriacy, M., 1997. Kinetic characterization of individual hexose transporters of *Saccharomyces cerevisiae* and their relation to the triggering mechanisms of glucose repression. Eur J Biochem. 245**,** 324-333.

Rieger, M., Kappeli, O., Fiechter, A., 1983a. The Role of Limited Respiration in the Incomplete Oxidation of Glucose by Saccharomyces-Cerevisiae. J Gen Microbiol. 129**,** 653-661.

Rieger, M., Käppeli, O., Fiechter, A., 1983b. The Role of Limited Respiration in the Incomplete Oxidation of Glucose by *Saccharomyces cerevisiae*. J Gen Microbiol. 129**,** 653-661.

Rigoulet, M., Aguilaniu, H., Avéret, N., Bunoust, O., Camougrand, N., Grandier-Vazeille, X., Larsson, C., Påhlman, I. L., Manon, S., Gustafsson, L., 2004. Organization and regulation of the cytosolic NADH metabolism in the yeast *Saccharomyces cerevisiae*. Mol Cell Biochem. 256**,** 73-81.

Roach, P. L., Clifton, I. J., Fulop, V., Harlos, K., Barton, G. J., Hajdu, J., Andersson, I., Schofield, C. J., Baldwin, J. E., 1995. Crystal-Structure of Isopenicillin N-Synthase Is the First from a New Structural Family of Enzymes. Nature. 375**,** 700-704.

Rogowska-Wrzesinska, A., Larsen, P. M., Blomberg, A., Gorg, A., Roepstorff, P., Norbeck, J., Fey, S. J., 2001. Comparison of the proteomes of three yeast wild type strains: CEN.PK2, FY1679 and W303. Compar Funct Genom. 2**,** 207-225.

Rytka, J., 1975. Positive Selection of General Amino Acid Permease Mutants in *Saccharomyces cerevisiae*. J Bacteriol. 121**,** 562-570.

Sakai, M., Ogawa, T., Matsuoka, M., Fukuda, H., 1997. Photosynthetic conversion of carbon dioxide to ethylene by the recombinant cyanobacterium, *Synechococcu*s sp. PCC 7942, which harbors a gene for the ethylene-forming enzyme of *Pseudomonas syringae*. J Ferment Bioeng. 84**,** 434-443.

Sato, M., Urushizaki, S., Nishiyama, K., Sakai, F., Ota, Y., 1987. Efficient Production of Ethylene by *Pseudomonas syringae* Pv Glycinea Which Causes Halo Blight in Soybeans. Agr Biol Chem Tokyo. 51**,** 1177-1178.

Sato, M., Watanabe, K., Yazawa, M., Takikawa, Y., Nishiyama, K., 1997. Detection of new ethylene producing bacteria, *Pseudomonas syringae* pvs. cannabina and sesami, by PCR amplification of genes for the ethylene forming enzyme. Phytopathology. 87**,** 1192-1196.

Serra, M. C. C., Mainar, A. M., Palavra, A. M. F., 2011. Solubility of Ethene in Water and in a Medium for the Cultivation of a Bacterial Strain. J Chem Eng Data. 56**,** 1596-1601.

Sims, R. E. H., Mabee, W., Saddler, J. N., Taylor, M., 2010. An overview of second generation biofuel technologies. Bioresource Technol. 101**,** 1570-1580.

Small, W. C., McAlister-Henn, L., 1998. Identification of a cytosolically directed NADH dehydrogenase in mitochondria of *Saccharomyces cerevisiae*. J Bacteriol. 180**,** 4051-4055.

Springham, D. G., Moses, V., Cape, R. E., 1999. Biotechnology : the science and the business. Harwood Academic Publishers, Amsterdam.

Stephanopoulos, G., Aristidou, A., Nielsen, J., 1998. Metabolic Engineering: Principles and Methodologies. Academic Press, San Diego, California.

Stephanopoulos, G., Vallino, J. J., 1991. Network Rigidity and Metabolic Engineering in Metabolite Overproduction. Science. 252**,** 1675-1681.

Takahama, K., Matsuoka, M., Nagahama, K., Ogawa, T., 2003. Construction and analysis of a recombinant cyanobacterium expressing a chromosomally inserted gene for an ethylene-forming enzyme at the psbAI locus. J Biosci Bioeng. 95**,** 302-305.

Tao, L., Dong, H. J., Chen, X., Chen, S. F., Wang, T. H., 2008. Expression of ethylene-forming enzyme (EFE) of *Pseudomonas syringae pv. glycinea* in *Trichoderma viride*. Appl Microbiol Biot. 80**,** 573-578.

Thiele, I., Palsson, B. O., 2010. A protocol for generating a high-quality genome-scale metabolic reconstruction. Nat Protoc. 5**,** 93-121.

Thomas, K. C., Spencer, M., 1978. Effects of Ethylene on Metabolism of *Saccharomyces cerevisiae*. Can J Microbiol. 24**,** 222-227.

Tilman, D., Socolow, R., Foley, J. A., Hill, J., Larson, E., Lynd, L., Pacala, S., Reilly, J., Searchinger, T., Somerville, C., Williams, R., 2009. Beneficial Biofuels-The Food, Energy, and Environment Trilemma. Science. 325**,** 270-271.

True, W. R., 2012. Global ethylene capacity continues advance in 2011. Oil Gas J. 110**,** 78-84.

Tyo, K. E., Alper, H. S., Stephanopoulos, G. N., 2007. Expanding the metabolic engineering toolbox: more options to engineer cells. Trends Biotechnol. 25**,** 132-137.

U.S. supreme court, Diamond v. Chakrabarty, 447 U.S. 303 (1980). No. 79-136, 1980.

Ungerer, J., Tao, L., Davis, M., Ghirardi, M., Maness, P. C., Yu, J. P., 2012. Sustained photosynthetic conversion of CO2 to ethylene in recombinant cyanobacterium *Synechocystis* 6803. Energ Environ Sci. 5**,** 8998-9006.

van Dijken, J. P., Bauer, J., Brambilla, L., Duboc, P., Francois, J. M., Gancedo, C., Giuseppin, M. L. F., Heijnen, J. J., Hoare, M., Lange, H. C., Madden, E. A., Niederberger, P., Nielsen, J., Parrou, J. L., Petit, T., Porro, D., Reuss, M., van Riel, N., Rizzi, M., Steensma, H. Y., Verrips, C. T., Vindelov, J., Pronk, J. T., 2000. An interlaboratory comparison of physiological and genetic properties of four Saccharomyces cerevisiae strains. Enzyme Microb Tech. 26**,** 706-714.

van Gulik, W. M., Heijnen, J. J., 1995. A Metabolic Network Stoichiometry Analysis of Microbial-Growth and Product Formation. Biotechnol Bioeng. 48**,** 681-698.

Van Hoek, P., Van Dijken, J. P., Pronk, J. T., 1998. Effect of specific growth rate on fermentative capacity of baker's yeast. Appl Environ Microb. 64**,** 4226-4233.

van Loon, L. C., Geraats, B. P. J., Linthorst, H. J. M., 2006. Ethylene as a modulator of disease resistance in plants. Trends Plant Sci. 11**,** 184-191.

van Urk, H., Postma, E., Scheffers, W. A., van Dijken, J. P., 1989. Glucose-Transport in Crabtree-Positive and Crabtree-Negative Yeasts. J Gen Microbiol. 135, 2399-2406.

Wang, F. F., Cui, X. K., Sun, Y., Dong, C. H., 2013. Ethylene signaling and regulation in plant growth and stress responses. Plant Cell Rep. 32, 1099-1109.

Wang, J. P., Wu, L. X., Xu, F., Lv, J., Jin, H. J., Chen, S. F., 2010. Metabolic engineering for ethylene production by inserting the ethylene-forming enzyme gene (efe) at the 16S rDNA sites of *Pseudomonas putida* KT2440. Bioresource Technol. 101, 6404-6409.

Varma, A., Boesch, B. W., Palsson, B. O., 1993. Biochemical Production Capabilities of *Escherichia coli*. Biotechnol Bioeng. 42, 59-73.

Varma, A., Palsson, B. O., 1994. Metabolic Flux Balancing - Basic Concepts, Scientific and Practical Use. Bio-Technol. 12, 994-998.

Warth, A. D., 1988. Effect of Benzoic-Acid on Growth-Yield of Yeasts Differing in Their Resistance to Preservatives. Appl Environ Microb. 54, 2091-2095.

Watson, J. D., Crick, F. H. C., 1953. Molecular Structure of Nucleic Acids - a Structure for Deoxyribose Nucleic Acid. Nature. 171, 737-738.

Watson, T. G., 1976. Amino acid Pool Composition of *Saccharomyces cerevisiae* as a Function of Growth rate and Amino acid Nitrogen source. J Gen Microbiol. 96, 263-268.

Weingart, H., Ullrich, H., Geider, K., Volksch, B., 2001. The role of ethylene production in virulence of *Pseudomonas syringae* pvs. glycinea and phaseolicola. Phytopathology. 91, 511-518.

Weingart, H., Volksch, B., 1997. Ethylene production by *Pseudomonas syringae* pathovars *in vitro* and *in planta*. Appl Environ Microb. 63, 156-161.

Weingart, H., Volksch, B., Ullrich, M. S., 1999. Comparison of ethylene production by *Pseudomonas syringae* and *Ralstonia solanacearum*. Phytopathology. 89, 360-365.

Vemuri, G. N., Eiteman, M. A., McEwen, J. E., Olsson, L., Nielsen, J., 2007. Increasing NADH oxidation reduces overflow metabolism in *Saccharomyces cerevisiae*. P Natl Acad Sci USA. 104, 2402-2407.

Wenger, J. W., Schwartz, K., Sherlock, G., 2010. Bulk Segregant Analysis by High-Throughput Sequencing Reveals a Novel Xylose Utilization Gene from *Saccharomyces cerevisiae*. Plos Genet. 6.

Verduyn, C., Postma, E., Scheffers, W. A., Van Dijken, J. P., 1992. Effect of Benzoic-Acid on Metabolic Fluxes in Yeasts - a Continuous-Culture Study on the Regulation of Respiration and Alcoholic Fermentation. Yeast. 8, 501-517.

Verduyn, C., Zomerdijk, T. P. L., Vandijken, J. P., Scheffers, W. A., 1984. Continuous Measurement of Ethanol-Production by Aerobic Yeast Suspensions with an Enzyme Electrode. Appl Microbiol Biot. 19, 181-185.

Wilmouth, R. C., Turnbull, J. J., Welford, R. W. D., Clifton, I. J., Prescott, A. G., Schofield, C. J., 2002. Structure and mechanism of anthocyanidin synthase from Arabidopsis thaliana. Structure. 10**,** 93-103.

Vitkup, D., Kharchenko, P., Wagner, A., 2006. Influence of metabolic network structure and function on enzyme evolution. Genome Biol. 7.

Worrell, E., Phylipsen, D., Einstein, D., Martin, N., Energy use and energy intensity of the U.S. chemical industry. Berkeley, CA, 2000.

Yang, S. F., Hoffman, N. E., 1984. Ethylene Biosynthesis and Its Regulation in Higher-Plants. Annu Rev Plant Phys. 35**,** 155-189.

Young, R. E., Pratt, H. K., Biale, J. B., 1951. Identification of Ethylene as a Volatile Product of the Fungus *Penicillium digitatum*. Plant Physiol. 26**,** 304-310.

Zampar, G. G., Kummel, A., Ewald, J., Jol, S., Niebel, B., Picotti, P., Aebersold, R., Sauer, U., Zamboni, N., Heinemann, M., 2013. Temporal system-level organization of the switch from glycolytic to gluconeogenic operation in yeast. Mol Syst Biol. 9.

Zhang, M. H., Yu, Y. Z., 2013. Dehydration of Ethanol to Ethylene. Ind Eng Chem Res. 52**,** 9505-9514.

Zhao, Q., Guo, H. W., 2011. Paradigms and Paradox in the Ethylene Signaling Pathway and Interaction Network. Mol Plant. 4**,** 626-634.

Zhou, P., Lugovskoy, A. A., Wagner, G., 2001. A solubility-enhancement tag (SET) for NMR studies of poorly behaving proteins. J Biomol Nmr. 20**,** 11-14.

Zomorrodi, A. R., Maranas, C. D., 2010. Improving the iMM904 *S. cerevisiae* metabolic model using essentiality and synthetic lethality data. Bmc Syst Biol. 4**,** 178.

Zupke, C., Stephanopoulos, G., 1995. Intracellular Flux Analysis in Hybridomas Using Mass Balances and in-Vitro C-13 Nmr. Biotechnol Bioeng. 45**,** 292-303.

Ågren, R., 2013. On metabolic networks and multi-omics integration. Chalmers University of Technology, Göteborg.

Österlund, T., Nookaew, I., Nielsen, J., 2012. Fifteen years of large scale metabolic modeling of yeast: Developments and impacts. Biotechnol Adv. 30**,** 979-988.