High expression of heterologous proteins by *Saccharomyces cerevisiae* grown on ethanol

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Hoge expressie van heterologe eiwitten door *Saccharomyces cerevisiae* gegroeid op ethanol

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. W.H. Gispen, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op maandag 18 september 2006 des middags te 2.30 uur

door

Antonius Martinus Johannes van de Laar

geboren op 19 december 1971 te Boxmeer

Promotor: **Prof. Dr. Ir. C.T. Verrips**

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Composition/information on ingredients
Synonyms
Ethyl Alcohol

CAS-No.:	64-17-5	EC-Index-No.:	603-002-00-5
M:	46.07 g/mol	EC-No.:	200-578-6
Formula Hill:	C_2H_6O		
Chemical formula:	C_2H_5OH		

Hazards identification
Highly flammable

Toxicological information
...
Further toxicological information
After inhalation of vapours: slight mucosal irritations. Risk of absorption.
After skin contact: After long-term exposure to the chemical: dermatitis.
After eye contact: Slight irritations
After swallowing of large amounts: nausea and vomiting.
Systemic effects: euphoria.
After absorption of large quantities: dizziness, inebriation, narcosis, respiratory paralysis.

Further data
The product should be handled with the care usual when dealing with chemicals.

From: MERCK Material Safety Data Sheets

Contents

Abstract

The production of Baker's yeast (*Saccharomyces cerevisiae*) for proofing dough is one of the oldest fermentation processes in human civilisation. It is characterized by the production of ethanol and carbon dioxide in the proofing process. Nowadays, *S. cerevisiae*, is also used for the production of recombinant proteins, which are of great importance for industrial applications. Mostly, these proteins are produced in high cell density fed-batch fermentation processes that use glucose as sole carbon source. However, a surplus of this sugar induces the production of ethanol. This ethanol fermentation has recently been analysed with DNA arrays, showing a remarkable large number of genes that are up or down regulated. Most of these changes are considered as undesirable and are called stress responses. Indeed stress proteins are induced and the yield of biomass on the carbon source is reduced.

To improve this production process the system was analysed in detail. Remarkably we found that cultivation on ethanol in the final phase of a simple batch fermentation process had a beneficial effect on the yield of several different heterologous proteins produced by *S. cerevisiae*. This process was further developed to fully exploit the potential of ethanol cultivation. The characteristics were first determined in batch and fed-batch experiments by investigating the influence of growth rate, oxygen concentration and ethanol accumulation level on the productivity of the process. This resulted in an improved production protocol.

Subsequently we investigated how *S. cerevisiae*, growing under stress conditions at a suboptimal use of energy managed to improve the yield. First, by focusing on the metabolic and physiological state of the yeast cell and then by studying the protein production and secretion processes in the cell. Several approaches were followed: Genome wide DNA arrays to get an overall picture of changes, the construction of a mutant of the heterologous product (variable domains of Camelid antibodies, VHH's) and electron microscopy (EM).

The genome wide DNA analyses confirmed that many processes were dramatically changed. Nitrogen and fatty acid metabolism play an important role by directing intermediates to the central metabolism to compensate for the carbon and energy shortage. A large up regulation of the catabolism of proteins was witnessed, however the ERAD response, normally up regulated in *S. cerevisiae* producing heterologous proteins was not up regulated. We also observed that in spite that the concentration of glucose in a fed-batch phase is practically zero, the *GAL7* promoter in front of the gene encoding the heterologous protein was much better transcribed. Also a very strong redox and oxidative stress effect was found. Mutant of the VHH's learned us that especially VHH's with folding problems due to the increased probability of illegal S-S bridge formation were much better produced with ethanol as carbon source than on glucose. However the improvement is not restricted to this phenomenon.

Finally the EM showed many remarkable features, most remarkably being the enrichment of the heterologous protein and the ER resident BiP in an enlarged cell wall.

On one hand these observations resulted in a completely new process for the production of heterologous proteins, which is now applied at industrial scale (> 10.000 L) on the other hand based on the results of the DNA arrays, mutant studies and EM observations we constructed qualitative models to explain the yield improvement.

CHAPTER 1

General introduction

Biotechnological aspects of heterologous protein expression

Introduction

Modern biotechnology has developed rapidly over the last 30 years since Cohen and Boyer developed molecular genetic techniques to introduce heterologous DNA into bacteria and to produce foreign proteins[1]. This knowledge opened the way for the production of heterologous proteins in industrial applications, which is now one of the major components of modern biotechnology. For pharmaceutical products these promises have been turned in reality, but for the market of consumer goods and industrial processes the number of applications remained relatively low.[2] As techniques in genomics and proteomics developed further, knowledge about the production of heterologous proteins increased. Consequently, production systems have improved drastically and more products have become profitable. Strains have been constructed in which metabolic routes are altered in favour of protein production[3]. Some strains are altered in morphology to make large scale cultivation easier[4] and the use of protease mutants[5], improved signal sequences, more efficient promoter systems, adapted cultivation techniques and recovery processes, make heterologous protein production increasingly cost effective[6]. So much so that the list of heterologous protein products on the various markets and in the pipeline is long[7].

In general there are several applications for heterologous proteins: the two main ones being for human health and industrial processing. In 2003, 75% of the biotech companies were engaged in health care applications while 12.8% worked on industrial related applications[8].
In table I an overview is given of some of the application fields and products related to heterologous protein production. The main products are biopharmaceuticals (active pharmaceutical ingredients) and industrial enzymes[6], but processing aids, food additives and animal health and feed are also growing application fields[2,8,9].

Table I.

Overview of main application areas related to heterologous protein production[8].

Application field	Application	Products
Human health	Prevention	Vaccines
	Diagnostics	Gene tagging
		Biosensors
	Therapeutics	Biopharmaceuticals
Industrial processing	Specialty chemicals	Amino acids
		Enzymes
	Food processing	Bioprocessing (enzymes)
		Vitamins
		Phytochemicals (nutraceuticals)

Of these markets the production of pharmaceutical products is in value by far the largest whereas in volume industrial detergent enzymes are dominant[10,11]. Table II shows an overview of heterologous industrial enzymes available on the market according to the Association of Manufacturers and Formulators of Enzyme Products (AMFEP) in 2004[11]. But a much longer list is available for pharmaceutical products on the market and in the pipeline. Up to 2004, 108 biotechnology medicines were approved by the FDA and on the market, while 324 were in development for 150 diseases, the majority of which are monoclonal antibodies[12].

The market can also be divided into its size or its cost price. 1995 Hensing[6] *et al.* described:

- Bulk proteins with a market size of 10^5-10^7 kg annually with a cost price \$5-\$500 /kg.
- Speciality proteins with 10^3-10^5 kg annually costing over \$100 /kg
- Medical analytical proteins 1-10^3 kg costing over \$1000 /kg

This survey showed that indeed for consumer goods and industrial applications high yields of a heterologous product are necessary to make such products economically feasible.

The specific application of the heterologous product requires an appropiate production system. And although each system has unique characteristics, they all have common factors that influence the choices to make for production of a suitable final product and for possible optimisations:

Product: What characteristics does the protein need to have and how much is required?

Host organism: What is the best-suited organism for a specific product and which strains are available for use?

Cultivation: What culturing technique fits the application best and what scale is necessary?

Application: What purification is needed and what concentration and quality should the final formulation have?

But basically, by choosing the host strain the other three factors are determined to a large extent.

In this thesis the optimisation of a heterologous protein production process is described. Therefore, different aspects of production systems are described to be able to study the specific process better and to find leads for production improvement. First, several host strains will be discussed. Then the production strain that is used in this thesis (*Saccharomyces cerevisiae*) will be described in more detail, focusing on the metabolic and protein production aspects. This is followed by a description of the cultivation methods. Then, an overview of production optimisation is discussed and finally the outline of this thesis is given.

Table II.

List of heterologous industrial enzymes and their applications. From: AMFEP (2004)[11]

The application abbreviations are explained in the legend. Feed refers to applications in livestock feed. Technical applications are non-food, detergent or industrial applications.

Principal enzymatic activity	Host organism	Donor organism	Applications		
			Food	Feed	Technical
Acetolactate decarboxylase (alpha)	Bacillus amyloliquefaciens or subtilis	Bacillus sp.	Bevr		
Aminopeptidase	Trichoderma reesei or longibrachiatum	Aspergillus sp.	Ches Egg Meat Milk Spic	Feed	Misc
Amylase (alpha)	Bacillus amyloliquefaciens or subtilis	Bacillus sp.	Bake Bevr Stch	Feed	Text
	Bacillus amyloliquefaciens or subtilis	Thermoactinomyces sp.	Bake	Feed	
	Bacillus licheniformis	Bacillus sp.	Bevr Stch Sugr		Dish Ldry Misc Pulp Text
Arabinofuranosidase	Aspergillus niger	Aspergillus sp.	Bevr		
Catalase	Aspergillus niger	Aspergillus sp.	Bake Bevr Ches Egg Fats Stch Sugr		Wast
Cellulase	Aspergillus oryzae	Humicola sp.			Ldry Pulp Text
	Aspergillus oryzae	Myceliopthora sp.			Text
	Aspergillus oryzae	Thielavia sp.			Text
	Trichoderma reesei or longibrachiatum	Trichoderma sp.		Feed	Text
Cyclodextrin glucanotransferase	Bacillus licheniformis	Thermoanaerobacter sp.	Stch		
Galactosidase (alpha)	Aspergillus oryzae	Aspergillus sp.		Feed	
	Saccharomyces cerevisiae	Guar plant		Feed	
Glucanase (beta)	Bacillus amyloliquefaciens or subtilis	Bacillus sp.	Bevr	Feed	
	Trichoderma reesei or longibrachiatum	Trichoderma sp.	Stch	Feed	Text
Glucoamylase or Amyloglucosidase	Aspergillus niger	Aspergillus sp.	Bevr Frut Stch		
Glucose isomerase	Streptomyces lividans	Actinoplanes sp.	Stch		
	Streptomyces rubiginosus	Streptomyces sp.	Stch		
Glucose oxidase	Aspergillus niger	Aspergillus sp.	Bake Egg		
Hemicellulase	Bacillus amyloliquefaciens or subtilis	Bacillus sp.	Bake Stch	Feed	
Laccase	Aspergillus oryzae	Myceliopthora sp.			Text
	Aspergillus oryzae	Polyporus sp.			Text
Lactase or Galactosidase (beta)	Aspergillus oryzae	Aspergillus sp.	Ches Diet Ice Milk		
	Kluyveromyces lactis	Kluyveromyces sp.	Ice Milk		
Lipase, triacylglycerol	Aspergillus oryzae	Candida sp.	Fats		Misc Text
	Aspergillus oryzae	Fusarium sp.	Bake Fats		
	Aspergillus oryzae	Rhizomucor sp.	Ches Fats Spic		Misc
	Aspergillus oryzae	Thermomyces sp.	Bake Fats		Dish Ldry Lthr Pulp
	Pseudomonas alcaligenes	Pseudomonas sp.			Ldry
Maltogenic amylase	Bacillus amyloliquefaciens or subtilis	Bacillus sp.	Bake Stch		
Mannanase (endo-1,4-beta)	Trichoderma reesei or longibrachiatum	Trichoderma sp.		Feed	
Pectate lyase	Bacillus licheniformis	Bacillus sp.			Text
Pectin lyase	Aspergillus niger var. awamori	Aspergillus sp.	Bevr Choc Frut	Feed	Text
	Trichoderma reesei or longibrachiatum	Aspergillus sp.	Bevr Choc Frut	Feed	Text
Pectin methylesterase or Pectinesterase	Aspergillus niger	Aspergillus sp.	Bevr Choc Frut	Feed	
	Aspergillus oryzae	Aspergillus sp.	Bevr Frut		Misc
	Trichoderma reesei or longibrachiatum	Aspergillus sp.	Bevr Choc Frut	Feed	Text
Penicillin amidase	Alcaligenes faecalis	Alcaligenes sp.			Misc
Phospholipase A	Trichoderma reesei or longibrachiatum	Aspergillus sp.	Bake Fats	Feed	Text
Phospholipase B	Trichoderma reesei or longibrachiatum	Aspergillus sp.	Bake Stch	Feed	
Phytase	Aspergillus niger	Aspergillus sp.		Feed	
	Aspergillus oryzae	Peniophora sp.		Feed	
	Trichoderma reesei or longibrachiatum	Aspergillus sp.		Feed	
Polygalacturonase or Pectinase	Trichoderma reesei or longibrachiatum	Aspergillus sp.	Bevr Choc Frut	Feed	Text
Protease (incl. milkclotting enzymes)	Aspergillus niger var. awamori	Calf stomach	Ches		
	Aspergillus oryzae	Aspergillus sp.			Lthr
	Aspergillus oryzae	Rhizomucor sp.	Ches		
	Bacillus alcalophilus	Bacillus sp.			Ldry
	Bacillus amyloliquefaciens or subtilis	Bacillus sp.	Bake Bevr Ches Fish Meat Milk Stch	Feed	
	Bacillus halodurans or lentus	Bacillus sp.			Dish Ldry Lthr
	Bacillus licheniformis	Bacillus sp.	Fish Meat		Dish Ldry
	Cryphonectria or Endothia parasitica	Cryphonectria sp.	Ches		
	Kluyveromyces lactis	Calf stomach	Ches		
Pullulanase	Bacillus licheniformis	Bacillus sp.	Stch		
	Bacillus subtilis	Bacillus sp.	Bevr Stch		
	Klebsiella planticola	Klebsiella sp.	Bevr Stch		
	Trichoderma reesei or longibrachiatum	Hormoconis sp.	Bake	Feed	
Xylanase	Aspergillus niger	Aspergillus sp.	Bake Bevr	Feed	
	Aspergillus niger var. awamori	Aspergillus sp.	Bake		
	Aspergillus oryzae	Aspergillus sp.	Stch		
	Aspergillus oryzae	Thermomyces sp.	Bake	Feed	
	Bacillus amyloliquefaciens or subtilis	Bacillus sp.	Bake Bevr Stch	Feed	Pulp Text
	Bacillus licheniformis	Bacillus sp.	Stch		Pulp
	Trichoderma reesei or longibrachiatum	Actinomadura sp.		Feed	
	Trichoderma reesei or longibrachiatum	Trichoderma sp.	Bevr Stch	Feed	Pulp

LEGEND

Food applications
Bake = Bakery
Bevr = Beverages (soft drinks, beer, wine)
Ches = Cheese
Choc = Cocoa, chocolate, coffee and tea
Conf = Confectionary
Diet = Dietary food
Egg = Egg
Fats = Fats and oils
Fish = Fish
Frut = Fruit and vegetables
Ice = Edible ice
Meat = Meat
Milk = Milk
Sald = Salads
Soup = Soups and broths
Spic = Spices and flavours
Stch = Cereal and starch
Sugr = Sugar and honey

Technical applications
Dish = Dishwashing powder
Ldry = Laundry
Lthr = Leather
Misc = Miscellaneous
Pulp = Pulp and paper
Text = Textile
Wast = Wastewater treatment

Host strains

All host organisms can be divided into three subclasses: higher eukaryotes, lower eukaryotes and prokaryotes.

Higher eukaryotes

Examples of heterologous protein producing higher eukaryotes are mammalian cell lines such as Chinese hamster ovary (CHO) cells, hybridomas, non-secreting myeloma cells[13,14] and insect cells[15]. They can produce highly dedicated complex human proteins in very specific and active forms[14]. In general these cell lines are less robust, as they are sensitive for CO_2 and metabolite accumulation, shear, stress and limitations in medium components and oxygen[7,13]. Therefore, defined expensive media are needed with a stricter quality control on the media components[16]. Also, to keep the culture in a productive condition, the cultivation techniques such as perfusion and wave bioreactors are more complex and scale up is difficult[7,15]. In addition, cultivations can take up to 60 or even over 100 days, which imposes heavy demands on culture stability[14]. With the still considerable yields and a high product value, the typical application area is the pharmaceutical market. With the extensive developments in for example molecular and computational techniques, these production systems are becoming increasingly efficient and robust[13].

Lower eukaryotes

Lower eukaryotes comprise species such as yeasts and moulds: e.g. *Saccharomyces, Aspergillus, Hansenula, Pichia* and *Kluyveromyces*. Selected species of these genera have been studied in detail and it has been shown that their protein production routes are very similar to higher eukaryotes including posttranslational modifications. Using recombinant DNA techniques, it has become possible to redirect the glycosylation in lower eukaryotes in such a way that the core is identical to the glycosylation pattern of human cells[17]. Thus proteins originating from higher eukaryotes can be successfully expressed in these strains without loosing activity or specificity. Due to their very efficient protein secretion mechanism high concentrations of extracellular heterologous protein can be produced. As these strains, especially *Saccharomyces cerevisiae*, have been known to man for many years, they are considered to be safe in food application (GRAS status) which makes approval of products produced in these systems by regulatory authorities, like the FDA less difficult. This all makes them especially suited for heterologous protein production in relatively cheap, high volumes and they often are used in food applications.[2,5,6,18-21]

Prokaryotes

Prokaryotes such as lactic acid bacteria, *Escherichia coli* and *Bacillus* species are less complex micro-organisms. Because of this and the genetic information available on these

organisms they were the first to be used widely in heterologous protein production applications[20,22]. These organisms are robust, transformants are stable, and they can be highly productive. The rapid growth, high cell densities[23] and cheap substrates make them easy to cultivate and scale up. A disadvantage of prokaryotes is their disability to perform posttranslational modifications, which makes many products not expressible in this system. Also, product secretion is often not efficient, which makes product recovery much more difficult[20,22,24]. Another very important factor is the fact that a lot of prokaryotes, or prokaryotic DNA fragments, are considered to be unsafe as pyrogens or pathogens and viral inclusions can be introduced in the final product[20]. Nevertheless they are used for the production of various pharmaceutical products[13] and industrial enzymes. Table III shows that in 2004, 31% of the total industrial enzyme production was performed with prokaryotes.

Total enzymes	186
Homologous product	64.5%
Heterologous product	35.5%
Aspergillus strains	36.6%
Trichoderma strains	10.8%
Penicillium strains	8.1%
Kluyveromyces strains	1.6%
Saccharomyces strains	1.1%
Prokaryotic strains	30.6%

Table III.
Distribution of industrial enzymes and production strains according to the AMFEP list in 2004[11]. Heterologous products are approximately one third of all the industrial applications, with *Aspergillus* and prokaryotic strains being the largest group of production organisms. *Saccharomyces* only accounts for 1% of the products in this list.

In this work we mainly discuss the heterologous protein production of Camelid antibody fragments (VHH) by the baker's yeast *Saccharomyces cerevisiae.*

The family of Camelids contains a set of IgG antibodies that are unique in that they are devoid of a light chain and have one single binding domain, referred to as VHH (figure 1)[25]. These single domain antibody fragments are very stable and show a high affinity and specificity. Furthermore they are easy to clone and express in a microbial host like *S. cerevisiae*[24,26]. VHH's find their application in affinity chromatography purposes in proteomic tools and bioprocessing.

We will therefore focus on *S. cerevisiae* and its production process, which besides VHH's also has been applied for the production of antifreeze peptides (AFP)[27] and industrial enzymes like cutinase[28,29] and α-galactosidase[30].

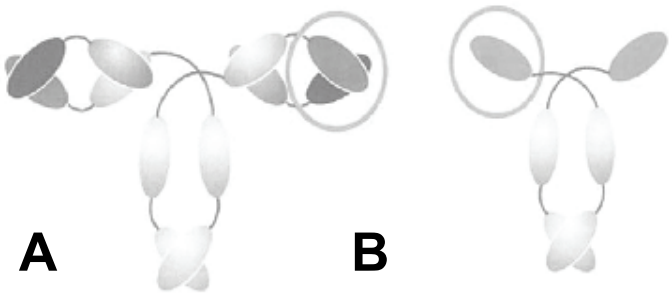

Figure 1.

Classical antibody (A) and a Camelid antibody devoid of light chain (B) with the variable domains encircled. As the variable domains are sufficient for many medical and nearly all industrial applications, the advantage of Camelid antibodies is obvious, the same binding properties with a single domain in stead of two domains.

Saccharomyces cerevisiae as production host

Due to its long history in brewing and baking *S. cerevisiae* is a GRAS organism and well characterised[19]. Since 1996 the whole genome sequence has been available[31] and extensive research on this eukaryotic model organism, keeps increasing the knowledge, also on biomolecular and cultivation techniques.

Many different industrial strains of *S. cerevisiae* have been developed with enhanced expression levels and the large scale fermentation processes are highly efficient[6] with high biomass concentrations and robust growth characteristics on cheap substrates. Furthermore, it performs post translational modifications and possesses an efficient secretion pathway making it suitable to produce a wide range of heterologous proteins in high quantities[6,19-21]. It is therefore one of the most popular hosts currently used in industry.

However, *S. cerevisiae* is not always optimal for the heterologous protein of interest. This yeast has a different glycosylation process than mammalian cells[32] and as secretion efficiency is among others depended on glycosylation, secretion might not be suitable[2]. In addition, *S. cerevisiae* is a Crabtree positive organism[33] causing it to go into a fermentative metabolism upon glucose accumulation, producing ethanol and decreasing growth and production yield. Besides that, glucose also induces catabolite repression under non-carbon limiting conditions that can also decrease the production efficiency[34]. The fact that *S. cerevisiae* is so well studied causes it sometimes difficult to use as property rights are being claimed for many features and techniques used.

In table IV a summary of the aspects of *S. cerevisiae* as heterologous protein production host are mentioned, in comparison with several other commonly used hosts: *Pichia pastoris*, *Hansenula polymorpha*, *Aspergillus niger* and *Aspergillus awamori* and *Kluyveromyces marxianus*. It shows that they have many aspects in common.

The production levels of *S. cerevisiae*, *H. Polymorpha* and *P. pastoris* have been shown to reach the same levels[6]. *Aspergillus*, however, has the greatest potential for extremely high heterologous protein production but still remains best suited for homologous products. This is also evident from the industrial enzymes listing (Table III and IV). These production levels are however heavily dependent on the properties of the protein that has to be produced.

The final factor determining the choice of host however is still a very practical one: the availability and property rights on the organisms and techniques. Companies have their house organisms which they favour and have property over and therefore a strong current development in optimising the production strain is focusing on finding alternative strains with more extreme properties[32].

Table IV.

Comparison of 4 common eukaryotic heterologous protein expression systems.

	Saccharomyces cerevisiae	*Hansenula polymorpha* *Pichia pastoris*	*Kluyveromyces marxianus*	*Aspergillus niger* and *awamori*
Key issue	Large knowledge base	Methylotrophic organism	Lactose substrates	Fungus, filamentous
GRAS	Yes	No	Yes	Yes
Knowledge	Longest history Genome sequenced Extensive research and database Modification techniques	Less known Genome recently sequenced Knowledge developing	Long history Modification techniques	Long history Genome sequenced Large knowledge
Carbon source	Glucose, molasses	Methanol	Lactose, whey	Glucose, molasses
Promoter	Strong promoters (*GAL*, *GAPDH*) Many alternatives	Very strong *MOX1* or *AOX1* promoter	Strong *LAC4* promoter Constitutive alternatives	*glaA* and others
Cultivation	Large scale Robust Efficient	Large scale Explosion proof facilities	Large scale	Large scale Possible filamentous growth
Positive aspects	Easy cultivation Extensive knowledge Developed techniques	Strong metabolic and secretion pathway Easy co expression	Low catabolite repression	Very high secretion (homologous)
Negative aspects	Intellectual property Crabtree positive Catabolite repression High glycosylation	Toxicity methanol Explosive carbon source Non GRAS	Fermentative metabolism possible (Pasteur effect)	Filamentous growth Heterologous less efficient Proteases
References	2,6,19-21,34	6,19,60-62	6,19	4,5,18,58,59

Carbon metabolism in S. cerevisiae

Although *S. cerevisiae* is able to grow on several carbon sources like fructose, galactose, ethanol, acetate and glycerol, the preferred one is glucose. Glucose can be transported into the cell by facilitated diffusion via a variety of sugar transporters and is subsequently metabolised in the cytosolic glycolysis to yield pyruvate and ATP and NADH. Pyruvate is subsequently metabolised in the mitochondria via acetyl-CoA and the citric acid cycle, also known as tricarboxylic acid cycle (TCA). The TCA intermediates are the main source for anabolic pathways to synthesise biomolecules like amino acids, fatty acids, purines, pyrimidines and others for maintenance, growth and (heterologous) protein production. The NADH generated by the metabolism is oxidised in the respiratory pathway in the mitochondria to yield ATP as central energy source and produce CO_2, H_2O from sugars.
However, this respiratory pathway is only effective under certain conditions: in the presence of oxygen, under glucose limitation and below growth rates close to the maximum growth rate.
First, *S. cerevisiae* is a facultative fermentative yeast, resulting in alcoholic fermentation in the absence of oxygen, yielding ethanol as main metabolic products[35]. Secondly, *S. cerevisiae* is a Crabtree positive organism, which is defined as the occurrence of alcoholic fermentation under aerobic conditions[33]. The 'long-term' Crabtree effect is the onset of ethanol production above a certain critical growth rate (approximately 0.25 h^{-1})[36]. The 'short-term' Crabtree effect is the occurrence of alcoholic fermentation upon excess sugar concentration in the medium. Both effects are due to the limited respiratory capacity in *S. cerevisiae*[37] which results in an overflow metabolism at the level of pyruvate. The carbon flux is directed towards acetaldehyde and subsequent formation of ethanol, which diffuses to the extracellular space. This aerobic alcoholic fermentation decreases the carbon and energy yield resulting in lower biomass concentrations and protein production.

In batch cultivation of *S. cerevisiae* on glucose as sole carbon source these metabolic properties are witnessed in an increase of the ethanol concentration due to alcoholic fermentation of glucose. When the glucose is depleted the cell is capable of consuming the ethanol as secondary substrate (ethanol oxidative growth), after a diauxic shift in which the enzyme pools and the carbon metabolism are adapted to the new situation[38] .
Ethanol diffuses passively into the cell and is converted into acetaldehyde, acetate and via acetyl-CoA further metabolised in the TCA. For the generation of saccharides, reserve carbohydrates, glycoproteins and nucleotides the gluconeogenesis pathway is induced, as the glycolysis is no longer active. Furthermore, to compensate for the retrieval of TCA intermediates for biosynthetic routes, and to increase the flux to pyruvate and the gluconeogenesis, the glyoxylate cycle is induced[39,40].
Through this adapted metabolism and the relatively lower carbon and energy content of ethanol it is a less favourable carbon source than glucose, and as a result growth rates and

biomass yield decrease[41]. Besides the metabolic aspects, ethanol invokes several other effects in the cell. It fluidises and permeabilises membranes thereby affecting ATPase activity[42] and it decreases cell viability, being considered toxic to the cell under higher concentrations[43]. It furthermore induces a strong stress response that resembles the response to heat shock[43,44]. Therefore, ethanol fermentation and subsequent oxidation are considered highly undesirable effects in *S. cerevisiae* cultivations, unless alcohol is the desired product in processes like bio-ethanol production and beer, wine and sake fermentations.

Another carbon source for *S. cerevisiae*, besides glucose and ethanol, is galactose. Galactose is transported into the cell by Gal2p and is subsequently metabolised via the Leloir pathway to glucose-6-phosphate, where it enters the glycolytic pathway. The genes of the galactose metabolism are induced by galactose, but strongly repressed when glucose concentrations are not limiting[34,45]. This glucose or catabolite repression is one of the key features of *S. cerevisiae* metabolism, as it not only regulates galactose metabolism but many more processes in the cell.

Heterologous protein production and the secretion pathway

For the production of heterologous proteins in *S. cerevisiae* it is essential to have a strong and high gene transcription and an efficient secretion mechanism (Figure 2).

Therefore the heterologous gene is placed in a production vector linked to a signal sequence which directs the transcript to the secretory pathway and behind a strong inducible promoter like *GAL7*. This vector is then introduced in the host strain, preferably in a high copy number (>20) and stable during mitosis so the production vector remains efficient even after many generations. Many of these vectors are episomal, but a multi copy rDNA integration vector has proven to be extremely stable and effective[46]. The rDNA is amplified upon exponential growth, so the heterologous gene also increases in copy number or at least is assured to have a stable presence.

In case of the *GAL7* promoter, the addition of galactose to the culture will induce gene transcription. The resulting mRNA is directed to ribosomes in the cytoplasm, where translation is initiated.

The nascent protein behind the signal sequence, has to be translocated over the membrane of the Endoplasmatic Reticulum (ER) to be further folded and processed. Depending on the signal sequence this can be done in a co-translational manner, where the protein is translocated while being synthesised. Or it is executed in a post translational manner, where the nascent protein is first synthesised and then translocated over the ER-membrane, being kept in its native form by chaperone proteins[47].

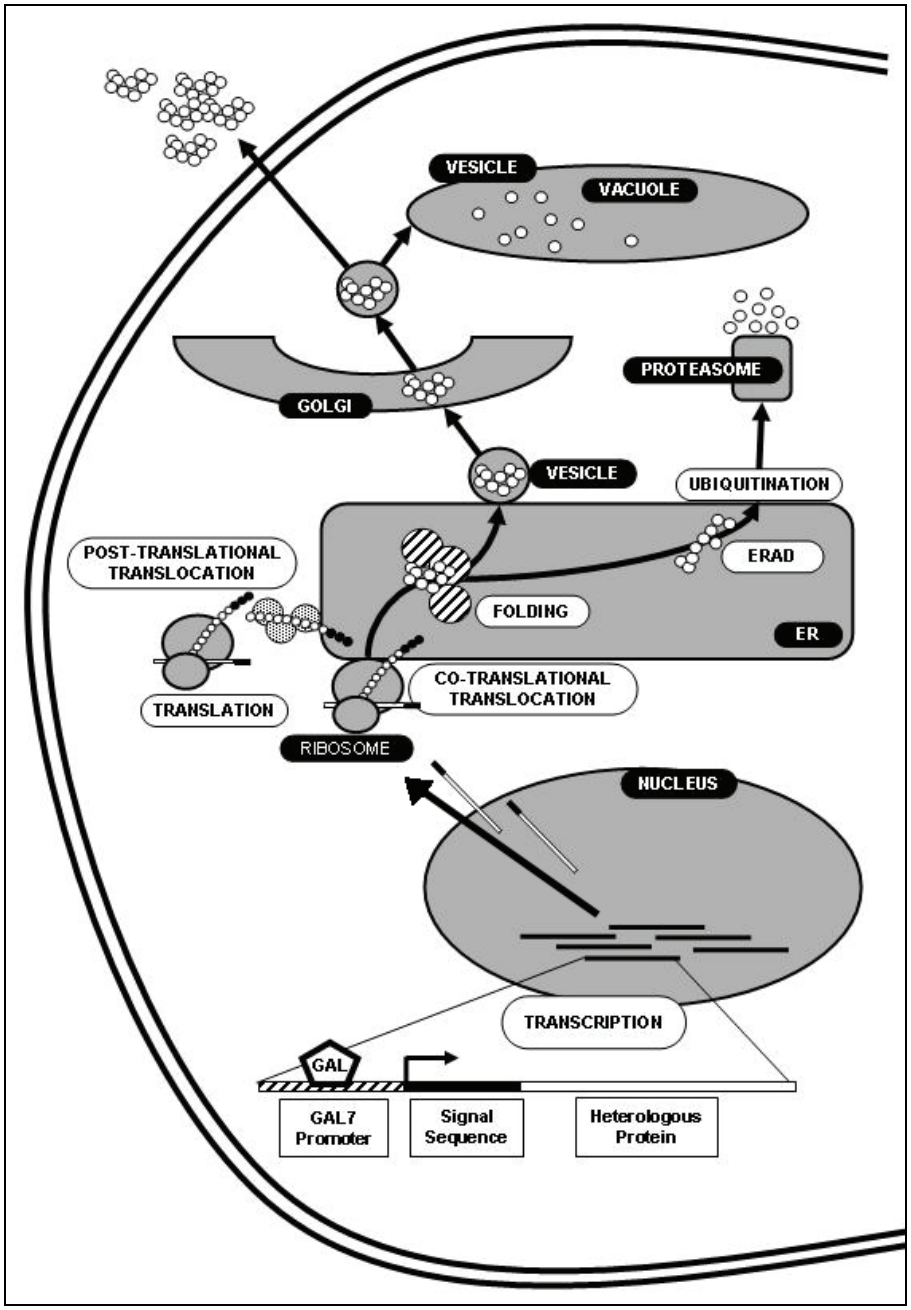

Figure 2.

General scheme of secretion pathway for heterologous protein production under a *GAL7* promoter. Upon galactose (GAL) induction the heterologous protein is transcribed behind a signal sequence. The resulting mRNA is transported to the ribosomes for translation and subsequent translocation into the endoplasmatic reticulum (ER) where the native protein is folded. Incorrectly folded proteins are designated for proteolysis in the proteasome by the ER associated degradation (ERAD) and ubiquitination. Correctly folded proteins are transported by vesicles to the Golgi apparatus for further processing and sorting. Finally, the protein can be secreted or targeted to the vacuole for proteolysis.

Inside the ER, the signal sequence is cleaved off by a peptidase and the protein is folded into its native conformation while undergoing post-translational modifications, glycosylation and the formation of intramolecular disulphide bonds. For an efficient processing of the proteins a large amount of chaperone proteins, foldases and enzymes like Protein Disulphide Isomerase

(PDI) and binding protein (BiP)[48]. Despite this extensive machinery the correct folding of proteins is not guaranteed, actually a large portion is destined for proteolysis after its folding state is incorrect. This ER associated degradation (ERAD) transports the incorrect folded protein out of the ER where it is labelled by ubiquitination and destined to the proteasome for degradation.

Correctly folded proteins are gathered in ER membrane vesicles and transported to the Golgi complex for further modifications, like additional glycosylation, and sorted for their final destination. For vacuolar proteins and molecules destined for degradation this is the vacuole, but the other main destination of the post Golgi transport is the cell membrane, either for cell wall proteins or secretion.

Culturing and production with *S. cerevisiae*

When a transformed and good producing *S. cerevisiae* strain is constructed, optimal cultivation is the next step towards industrial production.

The cultivation technique should give a high volumetric production and high product concentration of a stable product. The process should be reproducible and based on low cost manageable substrates[6] and fast, as process time is one of the main costs in large scale production.

In general there are three optional cultivation techniques: batch and fed-batch fermentation and continuous cultures (Figure 3).

Batch fermentation

This is a typical closed culture system with an initial and limited amount of nutrients[20]. The starting amount of glucose will be consumed at a maximum speed and so will growth rate be (Figure 3A). Due to the Crabtree effect[33] the ethanol concentration in the broth will increases and the glucose excess induces catabolite repression[6]. Therefore, when a galactose inducible promoter is used, the expression of the heterologous gene will be repressed until the glucose concentration has diminished.

In this system the growth rate can not be controlled which can be sub optimal for heterologous protein production and as the culture goes through the different growth phases, the conditions for protein production change. Towards the final phase of cultivation, inhibitory products accumulate, proteolytic activity increases and viability decreases[20]. In general these processes yield lower amounts of biomass and product because of the limited amount of substrate. On the other hand they take a relatively short time. Batch fermentations are not commonly used in industry with the exception of short, operationally flexible applications and small product volumes.

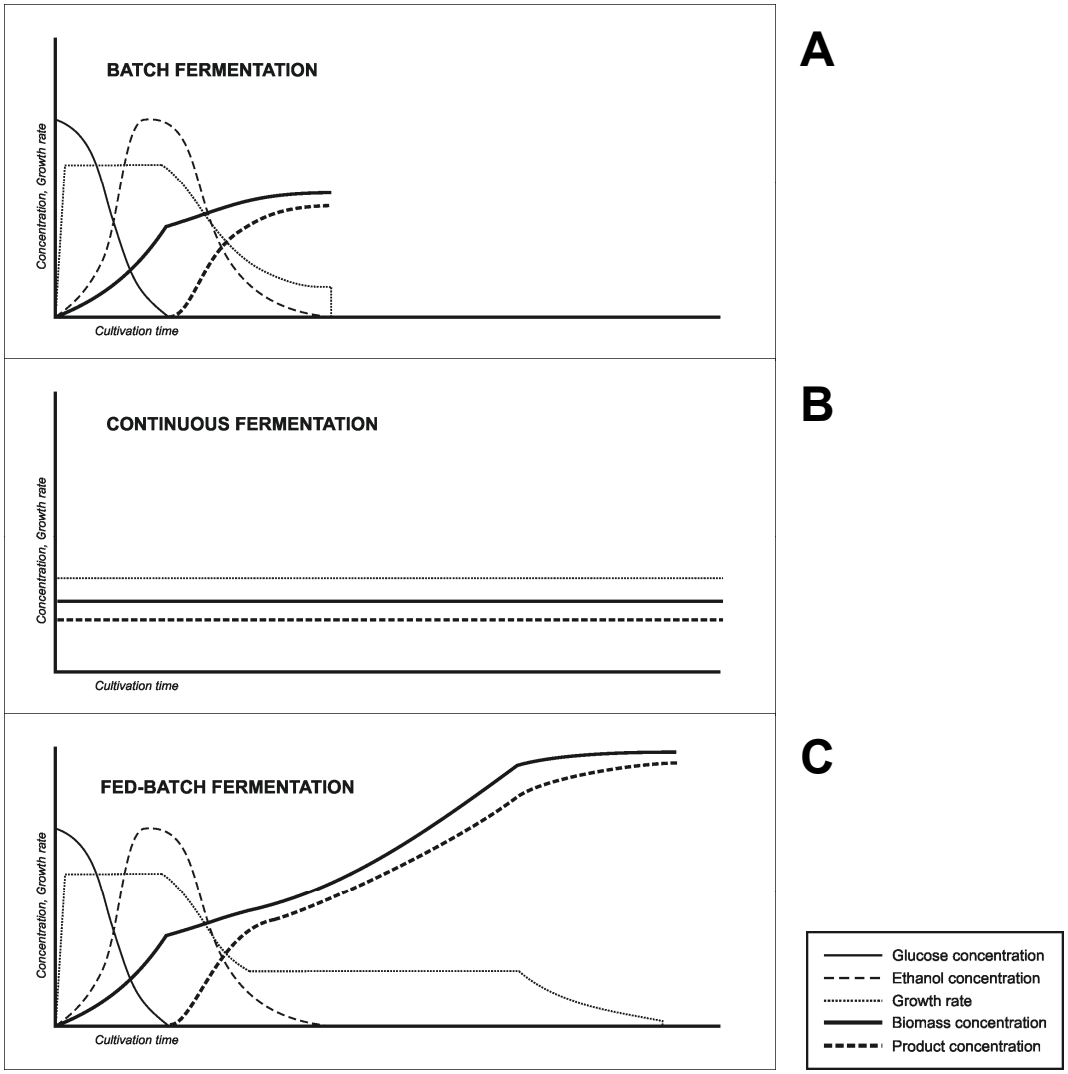

Figure 3.

Key characteristics of cultivation methods.

A: Batch fermentation. The glucose concentration decreases, ethanol is produced and subsequently consumed. The growth rate is maximum and decreases after the diauxic shift. Hence the lower biomass formation in the second part of cultivation. Under a *GAL7* promoter, the heterologous protein production starts when the glucose repression has disappeared. B: Continuous culture, steady state. The glucose (or ethanol) concentration is limiting and thus close to zero. The growth rate is maintained constant, regulated by the dilution rate. Biomass and product concentration also remain constant. C: Fed-batch cultivation. After the batch phase (figure 3A), the glucose feed is limiting the culture on a constant growth rate. Biomass and product concentration therefore further increase until oxygen transfer becomes limiting and growth rate and production decreases.

Continuous culture

The principle of this cultivation technique is to keep the culture in a steady state by feeding carbon source in such a rate that it determines the growth rate while at the same rate, culture broth is withdrawn from the system (Figure 3B). By doing this, the optimal conditions for heterologous protein production, such as growth rate, inducer concentration and oxygen level can be maintained while by-products can not accumulate. This yields a better overall

productivity than batch fermentations. There are several disadvantages to this system, especially for industrial applications. Product concentrations are low as it is cannot accumulate in the fermenter and volumes are very high due to the continuous effluent from the system. Also, these fermentations can last for relatively long time especially when the optimal growth rate is low and steady states are not reached within several days. This can harm the genetic stability of the production strain and therefore the productivity of the system. Another issue is the increased infection hazard with the diluted yeast culture and complex feeding regime, high volumes and long process time.[6,20,26,28,49] There are not many examples known of industrially applied continuous cultures and it is mainly used for research objectives.

Fed-batch fermentation

In a fed-batch fermentation system an initial batch phase is used for fast initial growth of biomass and a starting point for a controlled feed to the culture (Figure 3C). The feed will often consist of concentrated medium and an inducer to control heterologous protein production.

Depending on the application, the media can be defined or undefined. Mostly for low cost, high volume applications complex media are used, using molasses or glucose and yeast extracts but this may influence the recovery afterwards. Defined media are more expensive and often give lower yields but recovery is easier and the medium components are of better and reproducible quality.

The feed will be applied in such a way that the carbon source is limited to maintain an optimal growth rate in a pseudo steady state condition best suited for heterologous protein production. This also circumvents the Crabtree effect and catabolite repression. Besides this exponential feed profile, several other regimes are possible such as an RQ controlled feed to grow at maximum growth rate.

All other cultivation parameters like dissolved oxygen level, pH and temperature are controlled to maintain the optimal cultivation conditions. By continuing the feed, the total volume, biomass and product concentrations increase too high levels. This will increase oxygen uptake and heat production until at some point these parameters can no longer be controlled to the optimum level. Especially in large scale vessels these limitations are quicker to occur as the dissolved oxygen (DO_2) at some point can no longer be maintained as the power consumption for stirring in a stirred vessel has its maximum. As a result the oxygen concentration decreases and the growth rate will decrease. Also, cooling capacity is limited in large-scale vessels. When the metabolic heat production becomes too high, the temperature will increase and the growth rate has to be decreased and as a result of the sub-optimal conditions the productivity decreases. When net production is no longer profitable the fermentation will be terminated.

As high biomass and product concentrations can be reached in the fed-batch production method, volumetric productivity is the best of the three systems. Also, as the processes are shorter than continuous cultures, approximately 20-30 generations, strain stability and

contamination are much less of an issue than in continuous cultures. However, fed-batches with high biomass concentrations can have some negative side effects. The limitations towards the end can induce the accumulation of unwanted by-products and high CO_2 levels. Proteases in high amounts can degrade the product very rapidly. Also, the solubility of medium components and even the product of interest can be problematic at some point. This and for instance high viscosities can have a negative impact on further downstream processes[23]. However, fed-batch fermentations are by far the most commonly used production systems as they are the most productive per volume and time.

Downstream processing

The preferred heterologous protein production system so far described encompasses a *S. cerevisiae* secreting its product in high concentrations in a fed-batch fermentation on a rich medium. This makes product recovery easier than for instance intracellular products. In general recovery would therefore start with a biomass separation step. For *S. cerevisiae* this can be performed by several processes and the steps used often depend on the availability in the large-scale production plant. Micro-filtration, centrifugal separation, membranes and filters are commonly used for this. A next step would then be concentration of the broth as the large-scale volume is high and the product concentration is relatively low. A typical way to perform this would be ultra-filtration, where the product of interest is retained while smaller particles and water are lost on the permeate side. Other widely used techniques are precipitation and crystallisation. A final polishing or filtration step can further clean up the product. To isolate the product to high purity and concentration, chromatography steps will be necessary. This however is an expensive process and will greatly increase the production cost. Therefore, the downstream processing route also depends on the final application, more than any of the other aspects of heterologous protein production. In small scale process development and optimisation the downstream processes are often neglected while large amounts of products can be lost by not choosing the proper steps and conditions. This is mainly because downstream processing depends heavily on heuristics as Lightfoot describes in his review[50].

Optimisation of heterologous protein production

To increase the production of heterologous proteins several areas of the production system can be optimised. These areas can be divided in the biological and the technological aspects of heterologous protein production.

Biological optimisation

The biological optimisation can be applied to the production host or the vector system.
For instance the promoter system can be altered which, determines to the largest extent the expression level and also has an impact on media and culture conditions. A few main examples are given below:

a) Galactose regulated promoters are associated with the genes of galactose metabolism like *GAL7* [51]. In front of a heterologous protein encoding gene it makes a very powerful promoter system. It is however sensitive to glucose repression, which makes expression only possible under glucose limitations[21]. The cost of galactose as inducer is considerable and therefore *gal1* mutants have been created that can take galactose up but do not metabolise it[52].
b) Temperature controlled promoters can induce protein production upon a decrease in temperature[21]. In large-scale applications this will be difficult to apply. Temperature increase sensitive promoters would therefore be more applicable but not many applications have been described[6].
c) Phosphate regulated promoters are induced by inorganic phosphates but the use of complex media make this inducible promoter system impractical[6].
d) In addition, to these inducible promoters, there are also constitutive promoters such as *GAPDH* and *PGK*. Both are glycolytic promoters and glucose addition under some conditions can influence their activity. These promoters are capable of substantial activity and industrial applications are known[21]

Signal sequences are required to direct the heterologous protein towards secretion. Different signal sequences are being used in *S. cerevisiae* such as invertase (*SUC2*), mating factor alpha (*MFα1*) and acid phosphatase (*PHO5*)[53] but also synthetic ones have been generated, based on the general rules of amino acid sequences of effective signal sequences[54]. The signal sequence also determines the translocation to be co- or post translational which might affect the production efficiency[47]. Furthermore, each signal sequence can have its own secretion efficiency in combination with a specific heterologous protein.

Another factor in host selection and optimisation is the proteolytic activity. As precursors of proteolytic enzymes may accompany the heterologous proteins in the intracellular transport from the ER to the membrane this can result in internal breakdown of the product and thus

inefficient production. Also extracellular proteases can breakdown the already secreted heterologous protein in the culture medium. Not all heterologous proteins and *S. cerevisiae* strains are vulnerable to proteolytic breakdown to the same extent. Several contra measures can be taken: decreasing culturing temperature, add protease inhibitors (but because these compounds can be highly toxic this is often unwanted) or create protease mutants. As most proteases have been characterised this is well possible however other proteases can take over the function and too much mutations may cause the cell not to grow[20].

Glycosylation as posttranslational modification is inherent to protein production in both higher and lower eukaryotes. It is important for cell recognition, receptor binding, protein targeting, protein solubility and stability[21]. In *S. cerevisiae* O-linked glycosylation happens in the ER where mannoses are transferred to threonine and serine residues, which in general does not resemble higher eukaryotic O-glycosylation. N-glycosylation also starts in the ER linking sugars to asparagine residues. Then in the Golgi complex additional N-glycosylation can be performed and hyperglycosylation might occur in a much different pattern than that found in higher eukaryotes. If a secreted heterologous protein has a glycosylation pattern deviating from its original it can have adverse effects. It can cause an antigenic response in therapeutic applications or it can influence the protein folding and decrease the specific activity of the protein[21]. Also, as glycosylation by *S. cerevisiae* is not necessary homogenous the product might not be homogenous. To avoid harmful O-linked glycosylation, strains can be modified by mutations in for instance *PMT*-genes[55]. N-glycosylation can also be altered through several mutations[21].

Further modifications that can be performed to the production strain are metabolic engineering so strains are more efficient in carbon and energy use towards protein production[3] knowing that an estimated 25-40% of the genes are unnecessary for this cause [2]. Also genes directly involved in protein folding and secretion can be targeted for increased production like BiP in the ER[56]. But also on the level of the heterologous protein itself modifications can be done. Such as the removal of hydrophobic patches or introduction of an N-glycosylation site for better secretion[57] and/or adding tails for easier recovery.

Technological optimisation

To increase the overall productivity of a fed-batch fermentation the optimal conditions can be fine-tuned or controlled better. Also medium optimisation is powerful tool for increased productivity. Or, the costs of raw materials and operations can be decreased. Another approach is to increase biomass to extreme levels while balancing the productivity of the cells. By this method high cell density cultures have been developed where biomass concentrations can reach over 100 g/kg[23]. Additional techniques such as cell recycling while refreshing the medium, volume withdrawal and repeated fed-batches or logistic streamlining are all ways to increase the total productivity of fermentation.

Outline of this thesis

Based on the standard fed-batch process for the production of VHH antibody fragments in *Saccharomyces cerevisiae* optimisation studies were started to improve the general production. This is necessary because many different VHH's as well as other heterologous proteins should have a high enough productivity in this platform production system, to be economically feasible for industrial applications. The objective therefore is to screen the system for biological and technological leads for general optimisations.

In Chapter 2 the basic heterologous protein production system is analysed on both biological and technological factors to see where optimisations can be done for improving the product yield. This resulted in a change in carbon source that increased production dramatically, which offers a wide range of possible fermentation strategies to fully use this potential. Also an idea is formed as to the exact reason for this response to ethanol.

In Chapter 3 the optimisation of the fermentation on the alternative carbon source is described and technical key aspects are determined. In the end, a fermentation protocol is suggested that can be applied in an industrial environment. The effects are also shown to work for other heterologous products. These results suggest a strong dependency on cell status and the amino acid sequence of the heterologous protein.

In Chapter 4 the effect of ethanol as carbon source is more deeply investigated by DNA microarray analysis. Its purpose is to show how ethanol cultivation changes the cell state and which stress responses are evident.

In Chapter 5 the link between ethanol cultivation, cell status and improved heterologous protein production is investigated by further analysing the whole genomic response in combination with protein production kinetics and the cell physiology by electron microscopy. It shows that besides increased mRNA levels of the heterologous protein, also protein catabolism and an increased post Golgi secretion pathway are the main cause for the ethanol effect.

In Chapter 6, the discussion, the effects of ethanol on heterologous proteins production in *S. cerevisiae* are reviewed, and some recommendations are given.

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CHAPTER 2

Increased heterologous protein production by *Saccharomyces cerevisiae* growing on ethanol as sole carbon source

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Abstract

For the production of heterologous proteins, *Saccharomyces cerevisiae* is one of the most widely used organisms in glucose fed-batch fermentations. The productivity of this system can be optimised by strain and by fermentation improvement. For the optimised production of a Camelid antibody and an antifreeze protein this system was evaluated. In shake flask cultivations it was found that ethanol has a strong effect on productivity increase. Therefore glucose and ethanol fed-batch fermentations were compared. It appeared that specific heterologous protein production was up to five times higher in the ethanol cultivation and the main increase was witnessed in the final phase of the process. Through further analysis, glycosylation and growth rate were ruled out as the main reason for this increase in production. More likely the ethanol induced changes in metabolic and redox state, membrane and stress effects and protein folding in the endoplasmatic reticulum are the reason for the improved production. Although the true mechanism behind the ethanol effect on the observed increase in productivity remains unclear, it provides a powerful production system for heterologous protein production.

Introduction

Saccharomyces cerevisiae is one of the most commonly used eukaryotic host organisms for the production of heterologous proteins 1. The historical usage of this organism in the food industry is reflected in its GRAS status[2]. The combination of the large knowledge of yeast physiology and the fact that the yeast genome has been fully sequenced[3] has resulted in production strains which have been optimised for decreased protease activity[4], optimised glycosylation pattern[5,6,7] promoter systems[8], efficient signal sequences[9,10], and details of many aspects of metabolism[11]. In yeast biomass or heterologous protein production, fed-batch fermentations are most commonly applied to produce high amounts of biomass and/or product. For these processes mainly cheap and highly energetic sugar derivatives such as molasses or glucose are used as carbon and energy sources[2,12]. Oxidative carbon sources are thought to be unfavourable alternatives for these fermentative carbon sources for high yielding fermentation processes. Ethanol for instance is reported to inhibit growth and viability, affect transport, metabolism and membrane permeability and thus induce a stress response[13]. Although fed-batch fermentations of *S. cerevisiae* on glucose can give high yields of heterologous protein, typical large scale fed-batch fermentations are still limited in cooling capacity, oxygen transfer and handling of the large volume at the end of the fermentation process[2,14]. As a result of these limitations the biomass increase slows down and ethanol production can occur as a result of the Crabtree effect[15]. Moreover, glucose fermentations can also suffer from glucose repression at that stage, which can have a big impact on protein

expression levels. Especially when a glucose repressible induction system, such as galactose induced systems[16], is used for the expression of the heterologous product. Thus, in the fermentation process the phase with potentially the best production is not exploited fully. These productivity issues might be tackled by technical optimisation of the fermentation process but improvements can also be made by strain optimisation. To study the technical aspects in the fed-batch production process an analysis was made of *S. cerevisiae* fermentations on glucose in shake flask and fed-batch fermentation producing two quite different types of proteins. The first, VHH-BC15 is a 12 kD Camelid antibody fragment against human IgG. This VHH can be used as processing aid in immuno-affinity purification[17]. The second product is the antifreeze protein (AFP) type III HPLC12 from ocean pout, a 7 kD peptide[17,18] able to modify the ice crystallisation process. We used these two proteins because of their industrial importance and their quite different architecture (folding) and glycosylation. In this way the probability that we can extrapolate our findings to other heterologous proteins increases. During these studies we noticed an unexpected positive effect on the yield of heterologous proteins by growth on ethanol. Therefore, we used ethanol as an alternative carbon source and investigated the impact of ethanol on heterologous protein production in more detail.

Materials and methods

Strains

Saccharomyces cerevisiae strain CEN.PK102-3Agal1 (*gal1:URA3*, *leu2*, *ura3*) carried a multi copy integration vector, integrated at the rDNA locus[19], containing the protein coding sequence, linked to a signal sequence and under control of the *GAL7* promoter and *leu2d* selectable marker to maintain a high copy number. In table I the strains are described which produce the llama antibody fragment VHH-BC15 linked to the invertase *SUC2* sequence and to a synthetic signal sequence Hmss1. The same strain and vector were used for the production of AFP, but because of the heavy glycosylation of this protein we also studied production in an isogenic strain with a *pmt1* deletion.

Table I. Production strains and characteristics.

Product	Signal Peptide	Signal Sequence	Strain	Deletions
VHH-BC15	Hmss1	MKKLLLLLL------LLLLPANA	CEN.PK102-3Agal1	*GAL1*
AFP	SUC2	MMLLQAFLFL---LAGFAAKISA	CEN.PK102-3Agal1	*GAL1*
AFP	SUC2	MMLLQAFLFL---LAGFAAKISA	CEN.PK338 [1)]	*GAL1, PMT1*

1) Derived from CEN.PK102-3Agal1

Inoculum and shake flask cultivation

The inoculum was grown in a shake flask containing 50 ml Difco Yeast Nitrogen Base (YNB) for 48 hours at 30°C, 120 rpm and was subsequently transferred to 500 ml YPD medium (2% yeast extract, 1% peptone, 2% glucose) for 24 hour at 30°C, 120 rpm. Productivity experiments were performed in shake flasks with metal coils for increased aeration. They contained 500 ml YPD medium with 1 g/l galactose (Duchefa), and were inoculated with 30 ml full grown YNB culture and incubated (66 h, 30°C, 120 rpm).

Fed-batch fermentation medium

All fed-batch fermentations contained 5.5 L batch medium (22 g/kg glucose·1aq (Avebe), 10 g/kg yeast extract KatG (Ohly), 2.1 g/kg KH_2PO_4, 0.6 g/kg $MgSO_4 \cdot 7H_2O$, 0.4 g/kg Struktol J673 antifoam (Schill & Seilacher), 10 g/kg Egli trace metals[20], 1 g/kg Egli vitamins[20]. The feed medium of the glucose fed-batch fermentation contained 440 g/kg glucose·1aq (Avebe), 3 g/l galactose (Duchefa), 25 g/kg yeast extract KatG (Ohly), 12 g/kg KH_2PO_4, 2.5 g/kg $MgSO_4 \cdot 7H_2O$, 0.8 g/kg Struktol J673 (Schill & Seilacher), 20 g/kg Egli trace metals, 2 g/kg Egli vitamins.

The ethanol feed medium was separated in two equal volumes of nutrient and ethanol feed to avoid precipitation. The nutrient feed contained 6 g/l galactose, 50 g/kg yeast extract KatG, 24 g/kg KH_2PO_4, 5 g/kg $MgSO_4 \cdot 7H_2O$, 1.6 g/kg Struktol J673, 40 g/kg Egli trace metals, 4 g/kg Egli vitamins. The ethanol feed contained 670 g/kg ethanol (96.2% non-denatured, Lamers & Pleuger). The media were autoclaved for 25 minutes at 121°C, 1.2 bar (Linden autoclave). Sugars and vitamins were sterilised separately and the 67% ethanol solution was sterile.

Fermenter and growth conditions

The fed-batch fermentations were performed in standard bioreactors with a working volume of 10 litres. Dissolved oxygen (Ingold DO_2 electrode, Mettler-Toledo) was controlled by automatic adjustment of the impeller speed. The pH (Ingold Impro 3100 gel electrode, Mettler-Toledo) was controlled using 3 M phosphoric acid (Baker) and 12.5% v/v ammonia (Merck). Temperature (PT100 electrode) was controlled via a cooling jacket and cooling and heating fingers. The offgas was analysed (Prima 600 mass spectrophotometer, VG gas analysis systems) for ethanol concentration, rO_2 and rCO_2. The batch phase (30°C, 2 L/min air, DO_2 minimum 30%, pH 5.0) was started by inoculation with 500 ml full-grown YPD culture. The feed was automatically started when the ethanol concentration decreased below 300 ppm. In the feed phase (21°C, 6 L/min air) the exponential feed rate was applied to maintain a growth rate of 0.06 h^{-1}. In the glucose fermentation, the feed rate was set to linear when the DO_2 level decreased below 15%. In the ethanol fermentation the DO_2 decreased to 0% and subsequently accumulating ethanol increased to a maximum of 1500 ppm in the offgas. Then, to maintain this accumulation between 800 and 1500 ppm, a pulsed feed profile was applied.

Sampling and analysis

The VHH-BC15 concentration was determined by immuno-perfusion chromatography as described by Verheesen *et al*.[17] The chromatography matrix was prepared by coupling of 4.1 mg of human IgG Fc (ICN biochemicals) to 104 mg POROS EP (Perseptive Biosystems). 100 μl of the matrix was packed in a column (diameter 2.1 mm length 30 mm; Perseptive Biosystems, PEEK) using an HPLC system (Shimadzu). Samples were loaded on the column with a flow rate 0.5 ml/min in 3 minutes with equilibration buffer (1xPBS pH 7.4, Roche) and eluted with elution buffer (PBS adjusted with 4 M HCl to pH 2.1, Roche) in 1.5 minutes at a flow rate of 2 ml/min. Finally the column was re-equilibrated during 2.5 minutes at a flow rate of 2 ml/min. Detection was performed by UV adsorption at 214 nm. The AFP concentration was determined by reversed phase HPLC (Vydac Protein and Peptide C18 column). The running buffer was 0.06% TFA (Merck) solution in MilliQ, the elution buffer contains 0.054% TFA (Merck) and 80% acetonitrile (Baker) in MilliQ. Detection was performed at 214 nm and 280 nm. Glucose, ethanol and galactose were determined by HPLC using an HPX-87H Aminex column (300x7.8 mm, Biorad) at 30°C, using 5 mM sulphuric acid at a flow rate of 0.6 ml/min on a Hewlett Packard 1100 system. Biomass was measured by dry weight determination and expressed as g dry weight per kg fermentation broth. Product concentrations are expressed in units/l, reflecting a weight per volume concentration which is normalised to in-house developed protein standard solutions. The total amount of product was corrected for the biomass content, assuming a wet weight : dry weight ratio of 4. Total biomass (g) was expressed as dry weight concentration times the weight of the total fermentation broth. Total specific production (units/kg) was expressed as total product per total biomass.

Growth rates were calculated based on rO_2 data from offgas masspectrometric analysis. For this it was assumed that the yield of biomass on oxygen is constant during the fermentation. These assumptions were verified by calculation of the growth rate based on dry weight data. Because of the larger error in analysis (approximately 10%) of the dry weight data and the fewer data points, the rO_2 method was preferred for visualisation.

Results

Production system

In this study, we describe the production of two quite different heterologous proteins (i) the Camelid variable domain antibody VHH-BC15 and (ii) the antifreeze protein (AFP) from *Macrozoarces americanus*[21] by *S. cerevisiae* in glucose fed-batch fermentations. Factors contributing to the final yield of the heterologous product were analysed systematically, either experimentally or theoretically. The most important theoretically ones are discussed first. In this production system copy number of the heterologous gene and *GAL7* promoter induction can be considered to result in maximal transcription of the foreign gene into the corresponding mRNA[22]. Although the secondary structure of the mRNAs play a role in mRNA stability and efficiency of translation[23] there is still not sufficient knowledge how to optimise this. However, molecular modelling of the mRNA structures of VHH's did not reveal extremely unstable complexes, neither very long and stable hairpins that may impair translation (data not shown). For translation the codon usage is important and stretches of 3 or more codons corresponding to low abundant tRNA should be avoided[24]. To achieve maximal translation efficiency the total gene of AFP was synthesised chemically using preferred codons[21], similarly as we did for α–galactosidase[22]. However the gene encoding VHH-BC15 does not contain such stretches (data not shown) and therefore we used the original DNA sequence.

The next biological factor to be optimised was the signal sequence in front of the heterologous protein. Therefore we tested different synthetic signal sequences linked to of the VHH-BC15 antibody gene.

If secretion of the heterologous product causes problems, aggregates often accumulate in the ER and the yeast reacts by up regulation of a number of genes[25]. We have carried out DNA arrays of various combinations of strain, expression system and fermentation conditions. This will reported separately.

As AFP is heavily O-glycosylated when produced in *S. cerevisiae w*e tested the effect of deletion of a protein mannosyl transferase gene, *PMT1*, involved in O-glycosylation, on yield and functionality of AFP, the latter because glycosylation affected the functionality of AFP adversely.

Specific productivity in shake flask and fermenter

Based on an initial screening of the signal sequences, the sequence hmss1 was found to be an efficient signal sequence for translocation to the ER and therefore the production of VHH-BC15 with hmss1 as leader sequence was tested for growth under industrial fermentation conditions in a 10 L glucose fed-batch fermentation. In figure 1A the biomass (dry weights) of both the fed-batch fermentation and the shake flask are shown. Figure 1B also shows the VHH-BC15 production during both cultivations and finally the specific production is plotted in figure 1C. Note that the VHH-BC15 production in the fermenter starts after 12 h because the

batch phase is performed without galactose induction. The biomass concentration at the end of fermentation in the glucose fed-batch fermentation is much higher (101 g/kg) than that in the shake flask cultivation (10 g/kg). The product concentration and the total yield of VHH-BC15 are higher in the fed-batch fermentation, but the specific production in the shake flask cultivation is much higher than in the fermenter. Apparently, under the less controlled sub optimal conditions for growth in a shake flask, the cells are more productive.

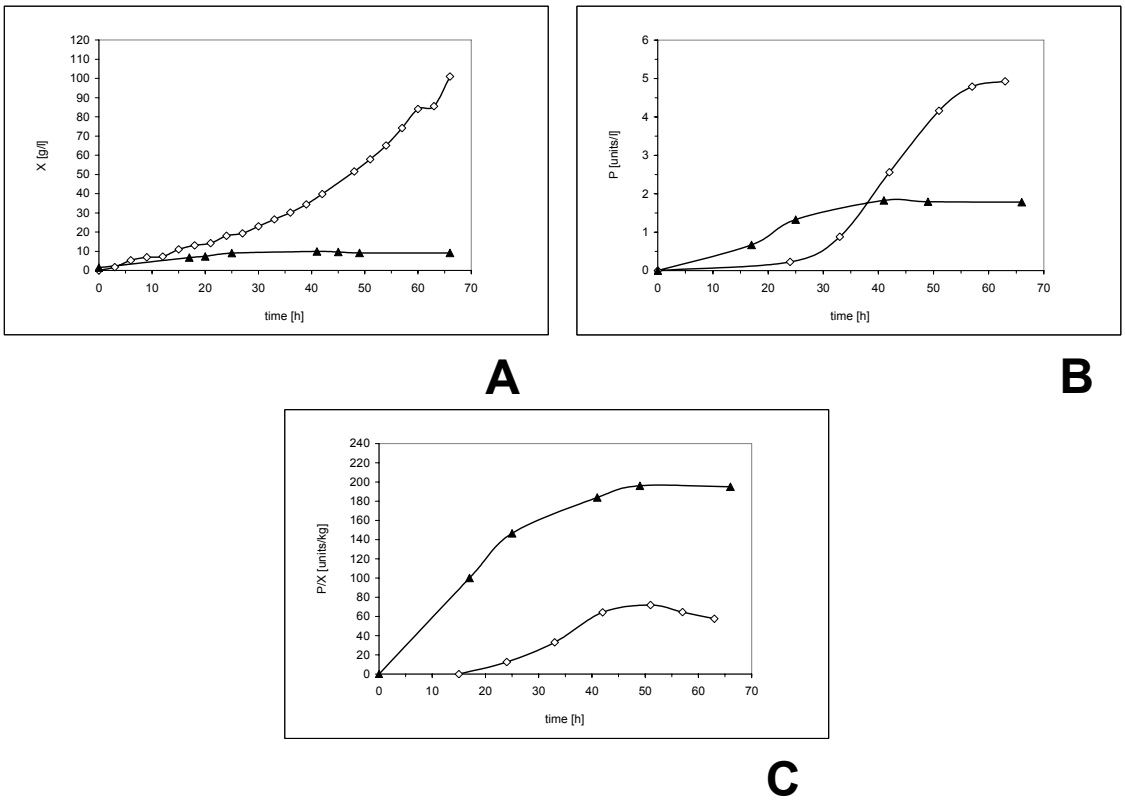


Figure 1.

Production characteristics of *S. cerevisiae* producing VHH-BC15 in shake flask (▲) and in glucose fed-batch cultivations (◇). A: dry weight concentration in time. B: product concentration in time. C: specific production calculated from concentrations in time.

The biomass and product concentration in a shake flask are lower than in a fed-batch fermentation, however, the specific production is considerably higher. Although a fed-batch fermentation produces much more total product, the efficiency of heterologous protein production is higher in shake flask cultivation.

In table II the carbon source, ethanol and inducer concentrations are shown for the first 24 hours of cultivation. This table shows that due to the Crabtree effect, the glucose is converted to ethanol[16,26] and thus that the actual VHH-BC15 production, which takes place after 15 hours, is under ethanol fermentation conditions instead of glucose as is the case during the fed-batch fermentation (figure 1C). This pattern is expected as during the beginning of the shake flask cultivation the glucose concentration is sufficient to result in repression of the *GAL7* promoter[27]. The fact however that after derepression the specific production is even higher than under glucose limiting conditions in a fed-batch fermentation is unexpected.

Moreover the conditions in the shake flask when the specific production is maximum are towards the end when ethanol is the sole carbon source, the dissolved oxygen concentration (DO_2) is minimal[28] and the growth rate is low. If these features can be successfully applied in an industrial fed-batch fermenter system, high specific and volumetric productions of heterologous proteins could be possible.

Table II.
Carbon source and inducer concentrations in shake flask cultivation of *S. cerevisiae* producing VHH-BC15 during first 24 hours.

Time (h)	Glucose (g/l)	Ethanol (g/l)	Galactose (g/l)
0	27.5	0	1.0
15	0	6.2	1.0
19	0	3.7	0.7
24	0	3.3	0.9

Glucose and Ethanol fed-batch fermentation

To investigate the effect of ethanol on production, we performed fed-batch fermentations with *S. cerevisiae* CEN.PK102-3Agal1 producing VHH-BC15 with ethanol as carbon source. The fermentation profiles with their distinctive phases are shown in figure 2. The batch phases for both glucose and ethanol fermentations are identical containing no galactose as inducer. In the exponential feed phase the applied growth rate for both was 0.06 h^{-1}, but in the ethanol fermentation the maximum oxygen consumption is reached earlier. In the final phase the glucose feed was applied linearly to avoid glucose accumulation in the broth which represses production and induces ethanol formation[16,27]. The DO_2 was therefore maintained above 10%. In the decline phase of the ethanol fermentation the feed was adjusted to maintain an ethanol accumulation level similar to that in the batch phase, between 800 and 1500 ppm, where 1000 ppm measured in the offgas is approximately 1% v/v in the broth. The DO_2 meanwhile decreased to 0%.

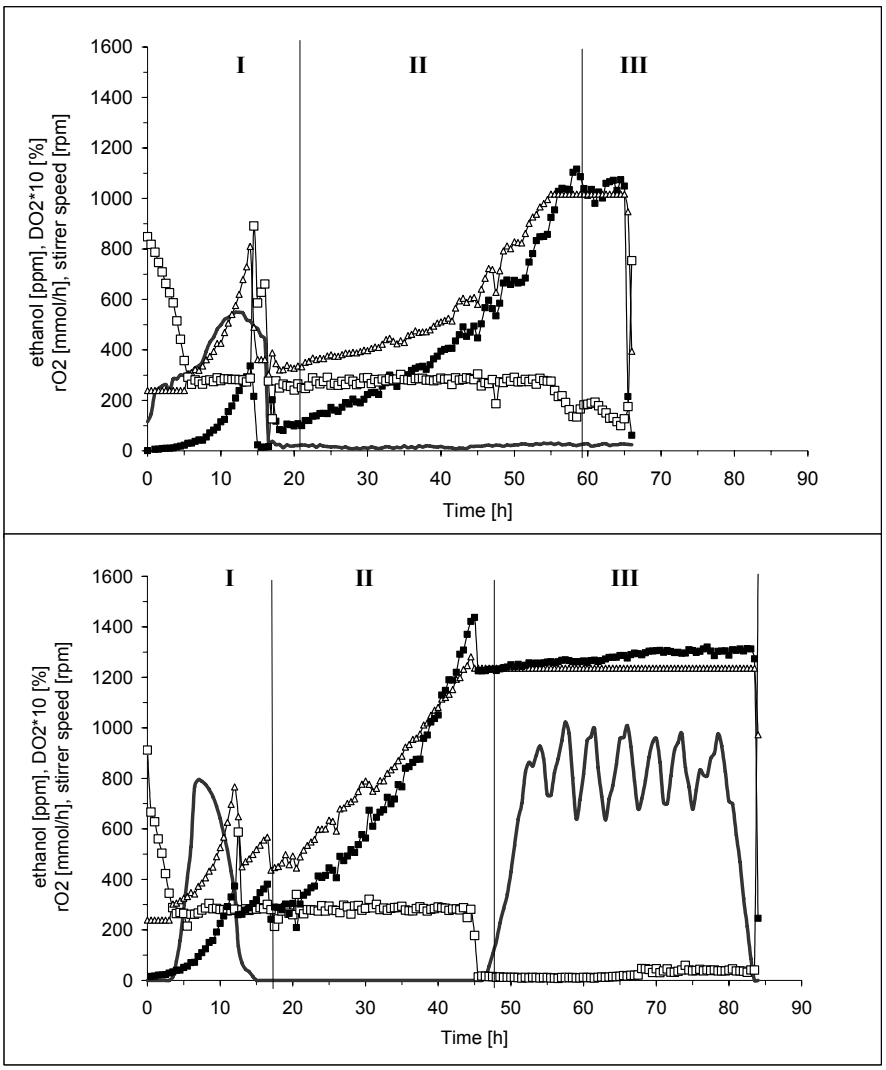


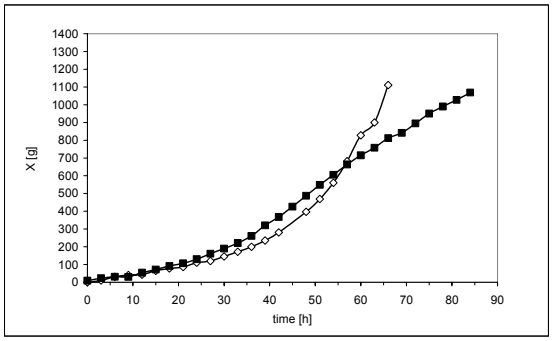
Figure 2.

Fermentation profiles of glucose (A) and ethanol (B) fed-batch fermentations with *S. cerevisiae* producing VHH-BC15. Ethanol ppm offgas (▬), DO_2*10 % (–□–), rO_2 mmol/h (–■–), Stirrer speed rpm (–△–).

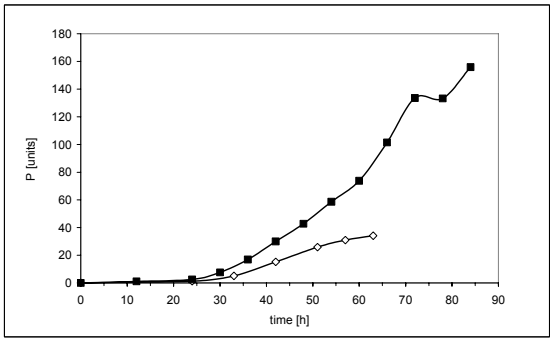
I: Batch phase on glucose. **II**: exponential feed phase on glucose or ethanol. **III**: Decline phase with linear glucose feed and decreased DO_2 or ethanol accumulation and oxygen limitation.

After the batch phase the limiting exponential feed is started, resulting in a carbon source concentration of approximately zero in the broth. As the oxygen consumption increases, so does the stirrer speed until its maximum value. In the glucose fermentation the feed rate is then set to linear to maintain a DO_2 above 10%. In the ethanol fermentation the DO_2 is allowed to decrease to 0% and ethanol accumulates in the broth to approximately 1000 ppm in the offgas, which is approximately 1%v/v in the broth.

The total biomass production and the total VHH-BC15 production are plotted in figure 3A and 3B. The ethanol fermentation was prolonged by adding more feed in the decline phase. The final biomass was therefore approximately the same as in the glucose fermentation. The VHH-BC15 production however is significantly higher in the ethanol fermentation. To normalise this production the specific production was calculated and plotted in figure 3C. It clearly shows that per amount of biomass, the VHH-BC15 production continues to increase while the production on glucose decreases as conditions get less favourable in the decline phase.



A



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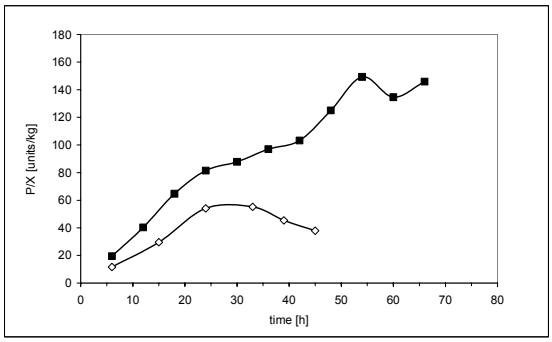

C

Figure 3.

Production characteristics of *S. cerevisiae* producing VHH-BC15 in fed-batch cultivations on glucose (◇) and ethanol (■).

A: total dry weight during cultivation. B: total product during cultivation. C: specific production in time, normalised to the start of VHH-BC15 production. On ethanol the biomass production is lower, especially in the final phase of the fed-batch process. The production however increases much stronger than in glucose fed-batch fermentation. This is clearly visible in the specific production plot where the ethanol cultivation is higher and remains increasing towards the end.

Table III shows the corresponding yields per phase. Furthermore the overall specific production in the glucose fermentation is only 30 units/kg dry mass while the ethanol process is almost 5 times more productive with 145 units/kg dry mass. This shows that this ethanol process for heterologous protein production is an improved production method but also has more scope in the final phase of fermentation.

Table III.

Yields per phase in glucose and ethanol fermentations *S. cerevisiae* producing VHH-BC15.

	Phase	End time (h)	Yxs[1)] (g/molC)	Ypx[2)] (units/kg)
Glucose	Batch	16		
	Exponential	55	15.80	48.75
	Decline	66	19.21	4.39
	Overall	66		30.67
Ethanol	Batch	13		
	Exponential	45	15.54	85.40
	Decline	84	11.64	111.82
	Overall	84		145.89

1) Yxs is calculated as dX/dS per phase where dS is the amount of mol C added as carbon source and dX is the increase in total biomass
2) Ypx is calculated as dP/X per phase with dP as the amount of product produced in that period and X as the total biomass at the end of that phase

To verify this ethanol effect, the same glucose and ethanol fermentations were performed at 21°C with another heterologous protein: AFP, a 7 kD peptide produced by an isogenic *S. cerevisiae* strain carrying an additional *pmt1* mutation. The integration vector is also similar but contains the invertase signal sequence. The fermentation profiles are shown in figure 4 and the production data is plotted in figure 5.

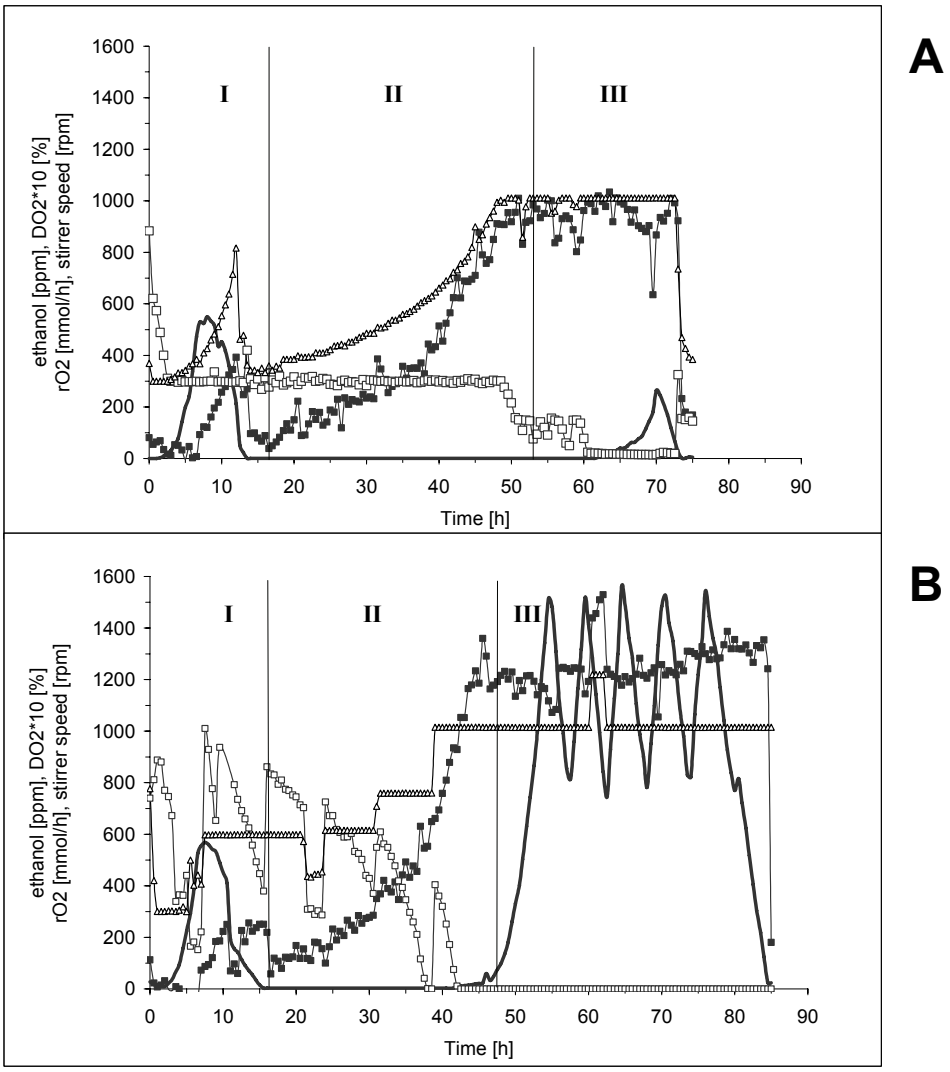

Figure 4.

Fermentation profiles of glucose (A) and ethanol (B) fed-batch fermentations with *S. cerevisiae* CEN.PK338 producing AFP. Ethanol ppm offgas ( ), DO_2*10 % ( ), rO_2 mmol/h ( ), Stirrer speed rpm ( )

I: Batch phase on glucose. **II**: exponential feed phase on glucose or ethanol. **III**: Decline phase with linear glucose feed and decreased DO_2 or ethanol accumulation and oxygen limitation.

Both profiles are similar to the ones described in figure 2. In the glucose fermentation some accumulation of ethanol at the end of cultivation can be seen as a result of the dissolved oxygen concentration that is near zero. The dissolved oxygen profile in the ethanol fermentation is slightly distorted and the ethanol accumulation level ranges form 800 to 1500 ppm in the offgas.

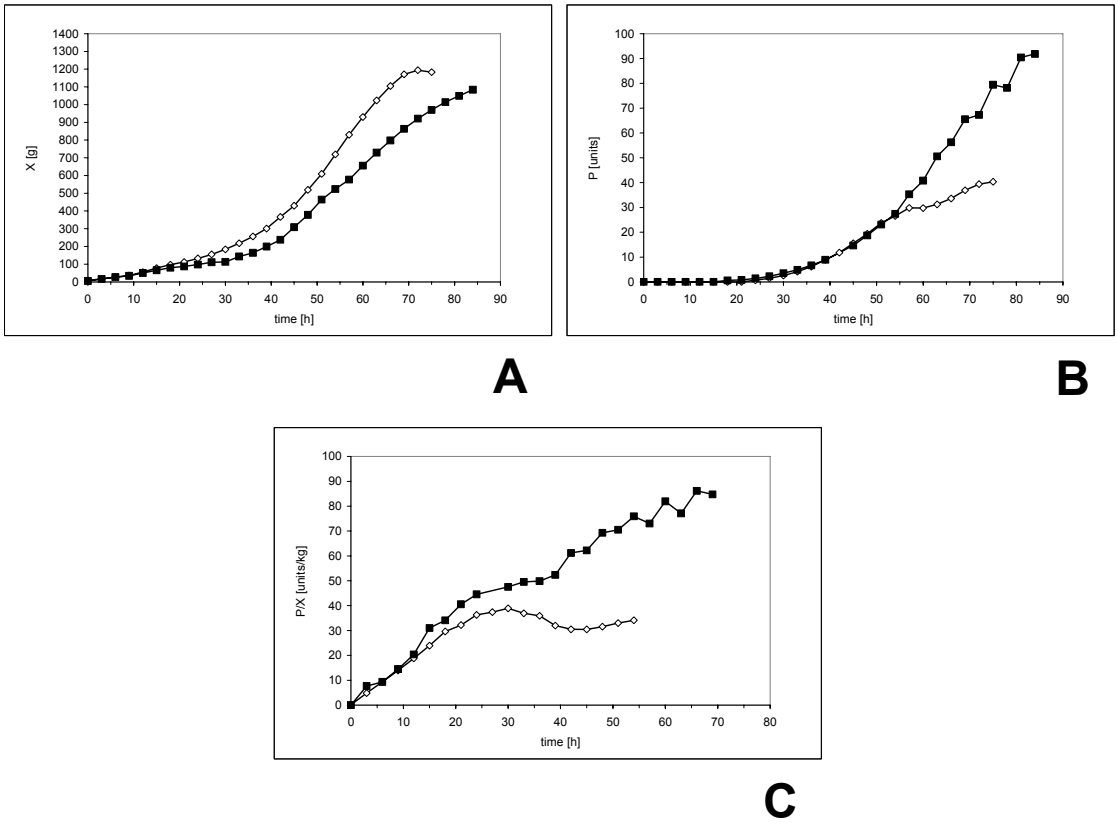

Figure 5.

Production characteristics of *S. cerevisiae* producing AFP in fed-batch cultivations on glucose (◇) and ethanol (■). A: total dry weight during cultivation. B: total product during cultivation. C: specific production in time, normalised to the start of AFP production.

The same profiles are seen with the production of VHH-BC15. Therefore the ethanol effect is witnessed in two different heterologous proteins.

Again, the results show that the specific production on ethanol is higher than on glucose, especially in the decline phase. This demonstrates clearly that the ethanol effect is not restricted to a particular class of heterologous proteins. In fact AFP is a quite different protein which is highly glycosylated when produced on glucose in *S. cerevisiae* and it is well described in the literature that glycosylation can affect the production yield of heterologous proteins. The amino acid sequence and the fermentation conditions are the most important factors in glycosylation[5,29].

AFP can be very heavily glycosylated while only the unglycosylated forms are quantified in the HPLC assay. Therefore the observed increase of heterologous product on ethanol growth could be due to a change in glycosylation and/or an artefact of the analysis. To rule this out, the production of AFP was compared in two isogenic strains, differing only in a mutation at the *PMT1* locus. Both strains show the effect of a higher product yield when grown on ethanol. Based on HPLC chromatograms the total amount of glycosylated and unglycosylated AFP was determined and the resulting glycosylation ratios in these fermentations are shown in figure 6. The effect of the *PMT1* deletion is obvious as the amount of unglycosylated AFP of

the total AFP secreted increased from 25% to 50%. There is however no significant difference in the ratio of unglycosylated product between glucose and ethanol grown cells and thus the observed increase in protein production on ethanol is unlikely to be caused by glycosylation effects.

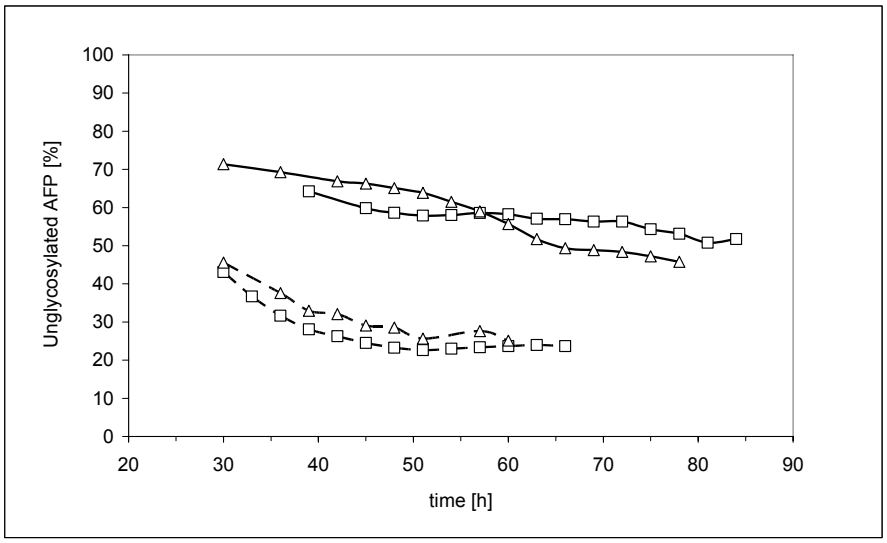

Figure 6.

Unglycosylated AFP ratio as function of fermentation time.

Glucose (△) and ethanol (□) fermentations with *S. cerevisiae* strain CEN.PK102-3Agal1 (- - -) and *pmt1* deletion strain CEN.PK338 (———).

It shows that there is no significant difference in glycosylation ratio between glucose and ethanol fed-batch fermentation. However, the deletion of the glycosylating *PMT1* gene increases production of the unglycosylated protein from 25% to 50-60% of the total heterologous protein.

The effect of ethanol on heterologous protein production could also be a growth rate effect. To verify this, the growth rates in the fed-batch fermentations on glucose and ethanol were plotted in time for both the VHH-BC15 and the AFP producing strain (figure 7A and 7B). As all fermentations were carried out with an applied growth rate of 0.06 h^{-1} in the feed phase the difference in specific production in that phase cannot be induced by the growth rate. But in the decline phase the growth rates decrease to about 0.03 h^{-1} for both ethanol and glucose. For VHH-BC15 (figure 7A) the glucose decline phase is stopped before this value is reached but the difference in protein production has started before the growth rate actually decreases (figure 3B and 3C). This is even more obvious in the AFP fermentations (figure 7B). Both growth rates on glucose and ethanol decrease simultaneously but the specific production is much higher for the ethanol fermentation (figure 5B and 5C). So the large difference in protein production on ethanol doesn't seem to be a direct result of a difference in growth rate.

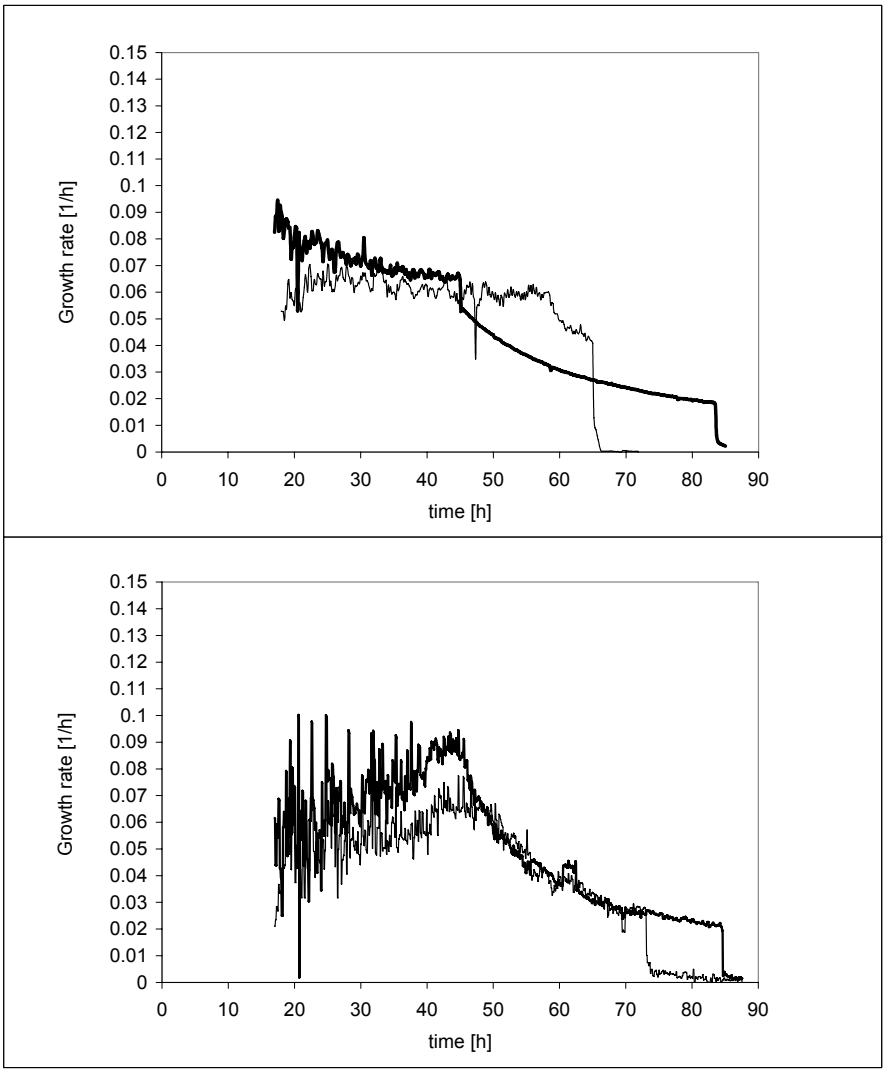

Figure 7.

Growth rates in ethanol (bold line) and glucose fermentations with *S.cerevisiae* producing VHH-BC15 (figure 7A) or AFP (figure 7B). Growth rate was calculated based on online rO_2 data. Similar graphs can be constructed based on dry weight data with a larger scatter.

The graphs show that for both heterologous proteins the same growth rate profile is similar for glucose and ethanol cultivation. Therefore it is highly unlikely that the large differences in specific production are induced by a growth rate effect.

Discussion

Optimisation of heterologous protein production can be achieved by altering both biological and technical factors. Using the multicopy integration system of which the defective *Leu2d* promoter ensures at least 20 copies and the *GAL7* promoter, we have optimised the transcription process[22]. The translation process can be optimised using optimal codons for yeast. Optimal signal sequences have been described in the literature[30] and we developed a completely synthetic one. For AFP, the degree of glycosylation proved to be an important

factor in the production of active (non glycosylated AFP) and therefore we inactivated the *PMT1* gene[31]. Further deletions affecting glycosylation were not possible as they impaired the growth *of S. cerevisiae* (results not shown).

Subsequently, the technical aspects of fermentation were optimised to improve the yield. As most industrial fermentations are carried out on molasses or other cheap sugars we started to investigate the effects of glucose on production of heterologous proteins. During these experiments we noticed that in the final phase of batch cultivation, when ethanol became the carbon source, higher specific yields of heterologous products were found than during growth on glucose. This surprising effect of ethanol as carbon source is especially strong in the decline phase in fed-batch fermentations when the oxygen transfer becomes limiting, ethanol accumulates and growth rate is decreasing.

Glucose repression has been reported many times to be detrimental for protein production in glucose systems[27,32]. This is however unlikely the reason for the observed effect in these experiments. Because in the glucose is the limiting carbon source in our fermentations its concentration in the broth is near zero and galactose is readily available. Under these conditions glucose repression should be absent[16,27] and galactose induction would therefore be maximal.

It has been reported that major changes in the metabolism of *S. cerevisiae* occur during diauxic shift, a normal phenomenon when *S. cerevisiae* is grown on high amounts of sugar in a batch process[33]. In addition to the fact that ethanol induces a heat shock like effect, it changes the redox balance in the cell. In particular, the first two steps in the conversion of ethanol into acetyl-CoA are typical redox processes[34]. It is well known that protein folding in the ER depends on the balance ATP:ADP and NADH:NAD in fact redox balances[35]. Under conditions of low growth rate, the energy generated by the conversion of ethanol can be used for a large part for the production of heterologous proteins, whereas the redox potential in the ER may favour the proper folding of these proteins. Whether an increase in proteins related to heat shock, in particular the cytoplasmic and ER chaperones also plays a role in this process remains to be elucidated.

In addition to the stress response, ethanol exerts many other physiological effects on the cells. It is described to be passively transported into the cell[36,37] which makes high ethanol concentrations a strong intracellular effector although its main target is the plasma membrane[38]. There it can have an effect on the membrane lipid composition[39] and it is able to permeabilise the membrane[38]. The results are a change in proton permeability, solute transport and thus a change in electrochemical gradient[38]. Rosa *et al.* described a change in ATPase activity in the plasma membrane as a result of ethanol[40]. These are all strong effects but none lead to a direct explanation of the increased protein production on ethanol.

Ethanol can induce a decrease in growth rate and the biomass yield is lower than on glucose partly due to the lower ATP yield. It has been reported that heterologous protein production is dependent on the growth rate[32,41]. However the results clearly showed that the increased production on ethanol appears under the same growth rates as in the glucose cultivations. Therefore the effect is most likely a combination of a more favourable redox potential and the increased levels of heat shock induced proteins[13,42].

The described ethanol effect can be an important tool to increase the heterologous protein yield in industrial fed-batch fermentations[43]. The most potent state of the fermenter, when biomass concentration is high and limiting conditions are present, can now be further used as protein production continues independent of growth. This efficient production under limiting conditions can also have a positive effect on up scaling these processes. With increasing scale the mixing times and the limitations in the micro environment of the cell get more important. The ethanol effect in these experiments is most pronounced under oxygen limitation and ethanol accumulation but the optimum process conditions to fully use this potential have to be further determined in fermentation research.

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CHAPTER 3

The optimisation of ethanol fed-batch fermentations for heterologous protein production in *Saccharomyces cerevisiae*

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Abstract

Saccharomyces cerevisiae is a widely used host organism for the production of heterologous proteins, often cultivated in glucose based fed-batch processes. This production system however has many factors limiting the productivity, mainly towards the end of the fermentation. A good alternative for this is the use of ethanol as sole carbon source, which yields increased production especially under the limiting conditions of the decline phase. To optimise an ethanol fed-batch process the production of Camelid antibodies and antifreeze proteins in this system were investigated. For that key characteristics such as growth rate and specific production in batch and fed-batch fermentations were determined under ethanol limitation and accumulation and growth limiting conditions in the final phase of the process. It appeared that an optimal production process should have an ethanol accumulation throughout the feed phase of approximately 1%v/v in the broth. Growth rates of 0.06 h^{-1} and high specific production are achieved by this method. The production remains very efficient even under growth rate decreasing circumstances. In such fed-batch processes the final limitations are the high biomass which affects proper mass transfer, the reduced amount of free water and the fact that at the end of the fed-batch the fermenter volume is fully occupied with broth. The productivity increase on ethanol versus glucose was also proven for several other Camelid antibodies some of which were heavily impaired in secretion on glucose, but very well produced on ethanol. This leads to the conclusion that the ethanol effect on improved heterologous protein production is linked to a stress response and folding and secretion efficiency.

Introduction

The productivity of a heterologous protein in a fermentation process to a large extent determines the cost of production. This productivity depends on the growth rate, biomass yield and specific production, which determine the length of the process and the final amount of biomass and product[1]. For microbial production systems, fed-batch processes using molasses or other cheap sugars as carbon sources are the current standard[2]. The process consists of a batch phase, a growth phase under controlled conditions and a decline phase at the end of cultivation[3].

These typical fed-batch processes have a number of technical and physical limitations mainly caused by the high biomass (up to 100 g/l and more) at the end of the process. In spite of optimised stirring, oxygen, heat transfer[2,3] and the homogenous supply of nutrients are impaired[4]. This results in both a decrease of the growth rate of the organism and the productivity[1]. In practice this means that the process is ended while large amounts of active biomass, capable of producing product, are present.

In previous work[5] we reported that *Saccharomyces cerevisiae* produced heterologous proteins quite well when grown on ethanol as a sole carbon source in fed-batch fermentations. The productivity was high, especially in the decline phase of the fermentation compared to the traditional glucose based fed-batch process.

The presence of ethanol causes dramatic changes in physiology and in the redox balance[6] of the yeast, resulting among others in an inhibition of the growth rate with increasing ethanol concentrations[7,8]. Ethanol also has been reported to cause a stress response[9,10], inducing heat shock proteins and it effects the permeability of membranes[11]. However, for both the Camelid antibody fragment VHH-BC15, recognising human IgG and the antifreeze protein type III HPLC12 (AFP), a higher specific production was established[5]. The fact that two quite different heterologous proteins are produced well with ethanol as sole carbon source indicates a more general principle underlying this observation.

In this study we investigated whether the observed yield increase of heterologous proteins when *S. cerevisiae* is grown on ethanol can be developed into a higher yielding fed-batch fermentation process. This was addressed by first determining key characteristics of ethanol fermentations, such as the impact of growth rates, the influence of the oxygen tension and the influence of ethanol accumulation. Based on the results, the impact on each phase of a fed-batch fermentation was investigated further to give an improved protocol for increased protein production. The optimised ethanol protocol was finally tested using several other heterologous proteins.

Materials and methods

Strains

Saccharomyces cerevisiae strain CEN.PK102-3Agal1 (*gal1:URA3*, *leu2*, *ura3*) carried a multi copy integration vector, integrated at the rDNA locus[12], containing the protein coding sequence, linked to a signal sequence and under control of the *GAL7* promoter and *leu2d* selectable marker to maintain a high copy number. In table I the strains are described which produce llama antibody fragments (VHH) and the antifreeze protein (AFP) linked to either the invertase (*SUC2*) signal sequence or a synthetic signal sequence Hmss1.

Inoculum

The inocula were grown in a shake flask containing 50 ml Difco Yeast Nitrogen Base (YNB) for 48 hour at 30°C, 120 rpm and subsequently transferred to 500 ml YPD medium (2% yeast extract, 1% peptone, 2% glucose) for 24 hour at 30°C, 120 rpm. The batch fermentations were inoculated by 500 ml YPD containing 1 g/l galactose (Duchefa) to fully induce heterologous gene expression before inoculation.

Table I.

Production strains and characteristics used in this work. The AFP producing strain contains an additional *PMT1* deletion to reduce glycosylation. Two types of signal peptides were used in these strains, invertase (*SUC2*) and a synthetic Hmss1. The VHH strains produce various VHH proteins that are highly similar in size and amino acid sequence. VHH-BC6CA is the same as VHH-BC6 with an additional cysteine and alanine residue at the C-terminal end.

Product	Strain	Deletions	Signal Peptide
AFP	CEN.PK338[1)]	*GAL1, PMT1*	SUC2
VHH-BC15	CEN.PK102-3Agal1	*GAL1*	Hmss1
VHH-BC6	CEN.PK102-3Agal1	*GAL1*	SUC2
VHH-BC6CA	CEN.PK102-3Agal1	*GAL1*	SUC2
VHH-BCF	CEN.PK102-3Agal1	*GAL1*	SUC2
VHH-BC5	CEN.PK102-3Agal1	*GAL1*	Hmss1

1) Derived from CEN.PK102-3Agal1

Batch and fed-batch fermentation medium

All fermentations contained 5.5 L batch medium: 22 g/kg glucose·1aq (Avebe), 10 g/kg yeast extract KatG (Ohly), 2.1 g/kg KH_2PO_4, 0.42 g/kg $MgSO_4$ 30%H_2O, 0.04 g/kg Struktol J673 antifoam (Schill & Seilacher), 10 g/kg Egli trace metals[13], 1 g/kg Egli vitamins[13]. The batch media for ethanol fermentations contained 1.8 g/l galactose. The feed medium of the glucose fed-batch fermentation contained 440 g/kg glucose·1aq (Avebe), 3 g/l galactose (Duchefa), 25 g/kg yeast extract KatG (Ohly), 12 g/kg KH_2PO_4, 1.75 g/kg $MgSO_4$ 30%H_2O, 0.08 g/kg Struktol J673 (Schill & Seilacher), 20 g/kg Egli trace metals, 2 g/kg Egli vitamins. The ethanol feed medium was separated in two equal volumes of ethanol feed (96.2% non-denatured, Lamers & Pleuger) and nutrient feed. The nutrient medium contained 75 g/kg yeast extract KatG, 36 g/kg KH_2PO_4, 5.3 g/kg $MgSO_4$ 30%H_2O, 0.24 g/kg Struktol J673, 60 g/kg Egli trace metals, 6 g/kg Egli vitamins. The extended fed-batch fermentations were carried out as normal ethanol fermentations with water from the batch and the feed medium being exchanged by ethanol. By this method 2.0 kg (1x), 2.4 kg (1.2x), 3.0 kg (1.5x), 4.0 kg (2x) and 4.72kg (2.4x) ethanol was fed. The other nutrients were increased with the same factors to assure that no nutrient limitations could arise. All the media were autoclaved for 25 minutes at 121°C, 1.2 bar and sugars and vitamins were sterilised separately.

Fermenter and growth conditions

All fermentations were performed in standard bioreactors with a working volume of 10 litres. Dissolved oxygen (DO_2) (Ingold electrode, Mettler-Toledo) was controlled by automatic adjustment of the impeller speed. The pH (Ingold Impro 3100 gel electrode, Mettler-Toledo) was controlled using 3 M phosphoric acid (Baker) and 2.5% v/v ammonia (Merck). Foaming was detected by a foam level sensor (Thermo Russell) and controlled by 5% Struktol J673 addition. Temperature (PT100 electrode) was controlled via a cooling jacket and cooling and

heating fingers. The offgas (Prima 600 mass spectrophotometer, VG gas analysis systems) analysed the ethanol concentration, oxygen consumption rate (rO_2 mmol/h) and carbon dioxide production rate (rCO_2 mmol/h). Adding 500 ml full-grown inoculum started the batch phase (30°C, 2 L/min air, DO_2 minimum 30%, pH 5.0). The exponential feed rates in both glucose and ethanol fermentations were automatically started when the ethanol concentration decreased below 300 ppm. The feed in fermentations with an ethanol accumulation was applied according to a pulsed feed profile to maintain the ethanol level within the demanded margins. All feed phases were performed at 21°C and 6 L/min airflow. In the glucose fermentation the feed rate was set to linear when the DO_2 level decreased below 15%. In the ethanol fermentations the DO_2 decreased to 0% and accumulated ethanol was further controlled by a pulsed feed profile.

Sampling and analysis

Ethanol accumulation was determined by offgas analysis by a mass spectrophotometer, as described above. The ethanol concentration is therefore expressed in ppm in the offgas, where a concentration of 1000 ppm in the offgas corresponds to approximately 1%v/v ethanol in the broth. However, to remain as close as possible to the analytical data, in this text ethanol concentrations will be expressed as ppm in the offgas.

The VHH-BC15 and other VHH concentrations were determined by immuno-perfusion chromatography as described by Verheesen *et al.*[14] The AFP concentration was determined by reversed phase HPLC[5]. Biomass (X) was measured by dry weight determination and expressed as g dry weight per kg fermentation broth. Product (P) concentrations are expressed in units/l, reflecting a weight per volume concentration which is normalised to in-house developed protein standard solutions. The total amount of product was corrected for the biomass content, assuming a wet weight over dry weight ratio of 4. Growth rates (μ, h^{-1}) were calculated based on rO_2 data from offgas masspectrometric analysis and verified by dry weight determination. For this it was assumed that the yield of biomass on oxygen is constant during the fermentation. These assumptions were verified by calculation of the growth rate based on dry weight data. Because of the larger error in analysis (approximately 10%) of the dry weight data and the fewer data points, the rO_2 method was preferred for visualisation.

Results

Batch fermentations

The previously described ethanol fed-batch fermentations gave a highest specific production in the decline phase where the growth rate decreases and ethanol accumulated in the broth[5]. To investigate the influence of this accumulation, four batch fermentations with different initial concentrations of ethanol were performed using *S. cerevisiae* producing AFP. Dissolved oxygen concentration was maintained above 30% and based on previous experiments we

concluded that medium components were not limiting. An overview of the results is shown in table II.

Table II.

Yields and consumption rate in ethanol batch fermentations. Four batch experiments were performed with a different starting concentration of ethanol in the medium. Also the corresponding ethanol in the offgas is given. The duration of the cultivation increased with concentration, while the biomass yield (Yxs) does not significantly change, but the ethanol consumption rate decreases. However, the product yield (Ypx) increases significantly with increased ethanol concentration, also witnessed in a high optimum for the specific productivity (qP) at 3200 ppm.

	Ethanol [1] (g/l)	Ethanol [2] (ppm)	time [3] (h)	Yxs[4] (g/Cmol)	qEtOH[5] (Cmol/g/h)	$EtOH_e$ [6] %	Ypx[7] (units/kg)	qP[8] (units/kg/h)
Batch 1	8.1	800	31	18.20	$1.547*10^{-3}$	2.1	45.67	1.47
Batch 2	15.5	1200	37	18.48	$1.338*10^{-3}$	21.7	61.55	1.66
Batch 3	32.8	3200	57	17.99	$0.919*10^{-3}$	42.2	104.11	1.83
Batch 4	65.7	8000	100	16.02	$0.593*10^{-3}$	62.6	117.86	1.18

1) Initial concentration in batch fermentation
2) Initial concentration measured in offgas
3) Total fermentation time
4) Yxs: yield biomass on substrate. Calculated by dX/dS.
5) qEtOH: specific ethanol consumption rate, corrected for ethanol evaporation. Calculated by dS/(dX*dt)
6) $EtOH_e$: evaporated ethanol as percentage of initial amount
7) Ypx: yield product on biomass. Calculated by dP/X
8) qP: specific productivity. Calculated by dP/(X*dt)

With increasing ethanol concentration the biomass yield (Yxs) remained constant until a significant decrease was seen at the highest level of ethanol (65.7 g/l). Apparently at this concentration the ethanol more strongly influences the physiological state of the cell. A methylene blue colouring test verified that the cells under all conditions were still viable as was reported earlier[7] (data not shown). In figure 1 the growth rates for each fermentation are plotted. It shows that the growth rate decreased as the cells were subjected for a longer period to the ethanol levels. Also, a higher concentration of ethanol seems to decrease the growth rate further, especially above an initial ethanol concentration of 15.5 g/l. These data clearly illustrate the growth inhibiting effects of ethanol[15]. This effect is also confirmed in the significant decrease of the ethanol consumption rate (qEtOH) in the same experiments (table II).

To account for the loss of carbon source by evaporation of ethanol in these batch-fermenter cultivations, the amount was calculated and verified by masspec analysis of the offgas. The quantity of substrate evaporation is significant and in batch 4 is almost 63% as a result of the higher concentration and longer cultivation time.

Unlike the biomass yield, the product yield (Ypx) increased substantially with elevated ethanol concentrations, up to a 2.5-fold maximum as shown by the specific production plot (figure 2).

But, although the specific production increased, the total productivity (qP) of the batch 4 fermentation was much lower (table II). At 21°C and with ethanol concentrations around 15 g/l

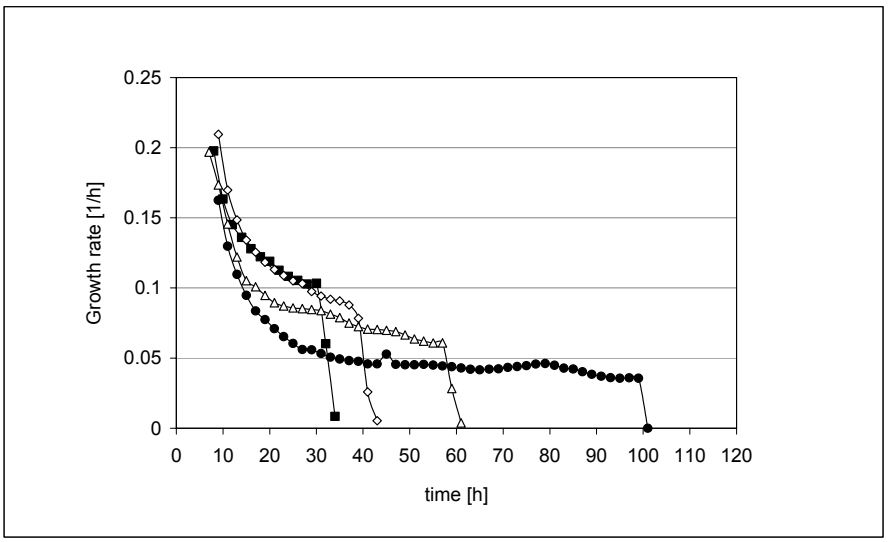

Figure 1.

Growth rates during batch fermentations on ethanol as sole carbon source. Four batch fermentations were performed with different initial ethanol concentrations: 8.1g/l (■), 15.5g/l (◇), 32.8g/l (△), 65.7g/l (●). The growth rate for each fermentation was calculated based on rO_2 offgas data. The higher ethanol concentrations decrease the growth rate.

however high production levels are obtained at growth rates above 0.06 h^{-1}, which was determined to be the optimal growth rate for heterologous protein production in the *S. cerevisiae* CEN.PK102-3A gal1 production system in glucose fed-batch fermentations (data not shown). These results suggest that with ethanol in a fed-batch fermentation higher growth rates and productivity are achievable.

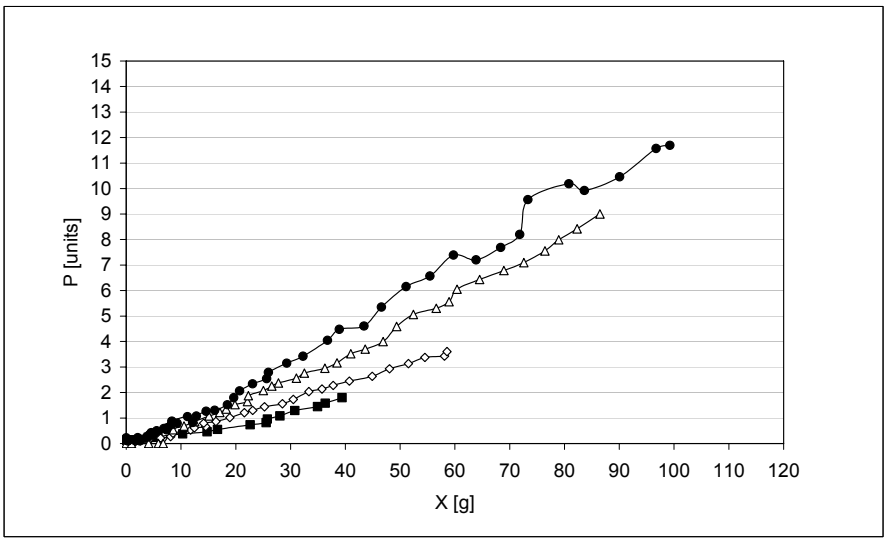

Figure 2.

The total AFP production (P) plotted versus the total biomass (X) for four batch fermentations with different initial ethanol concentrations: 8.1g/l (■), 15.5g/l (◇), 32.8g/l (△) and 65.7g/l (●). The slope of the graph reflects the total specific production, which increases with higher ethanol concentrations.

Fed-batch fermentation design

The described batch fermentations clearly showed that ethanol accumulation could have a positive effect on production even at growth rates above 0.06 h^{-1}. Based on these results the fed-batch process for the production of VHH-BC15 was optimised by analysing the influence of the growth rate, the amount of ethanol accumulation and the DO_2 in both the exponential and decline phase. In table III the results of these experiments are given.

Table III.

Yields and key results in the exponential phase and the decline phase for all fed-batch fermentations performed. The results for the two fermentation phases were calculated separately. In the ethanol limited exponential phases the applied growth rate is depicted, as well as the duration of the phase, the increase in total biomass and product. Based on these data the product yield and specific productivity were calculated.

Exponential phase							
Cultivation	Applied μ (h^{-1})	Ethanol level [1] (ppm)	dt [2] (h)	dX[3] (g)	dP[4] (units)	Ypx[5] (units/kg)	qP[6] (units/kg/h)
Glu 0.06	0.06	0	39	530	29.2	48.8	1.25
EtOH 0.06–1000	0.06	0	32	366	36.4	85.4	2.67
EtOH 0.03	0.03	0	73	629	56.3	77.04	1.06
EtOH 500-500	-	500	33	358	44.2	110.5	3.35
EtOH 1000-1000	-	1000	40	498	78.0	145.8	3.64
EtOH 1000-4000	-	1000	42	505	95.0	172.7	4.11
EtOH 2000-4000	-	2000	52	493	97.2	180.3	3.47
Decline phase							
Cultivation		Ethanol level [1] (ppm)	dt [2] (h)	dX[3] (g)	dP[4] (units)	Ypx[5] (units/kg)	qP[6] (units/kg/h)
Glu 0.06		0	11	512	4.88	4.39	0.40
EtOH 0.06–1000		800-1400	39	642	119.48	111.82	2.87
EtOH 0.03		0	21	442	80.68	68.79	3.28
EtOH 500-500		500	41	695	115.37	105.39	2.57
EtOH 1000-1000		1000	31	620	126.61	109.64	3.54
EtOH 1000-4000		4000	32	558	63.45	57.25	1.79
EtOH 2000-4000		4000	33	480	95.49	93.72	2.84

1) Ethanol concentration in ppm in offgas
2) dt: duration of the fermentation phase
3) dX: total biomass increase in dry weight in fermentation phase
4) dP: total product increase in fermentation phase
5) Ypx: yield product on biomass. Calculated by dP/X
6) qP: specific productivity. Calculated by dP/(X*dt)

Carbon limited exponential growth

The growth rate in the exponential feed phase is of major importance for the productivity of the fermentation[16,17]. In previous work the glucose and ethanol limited fed-batch fermentations were performed with a growth rate of 0.06 h^{-1} in the exponential phase as that proved to be the best growth rate for optimal production of heterologous proteins[5].

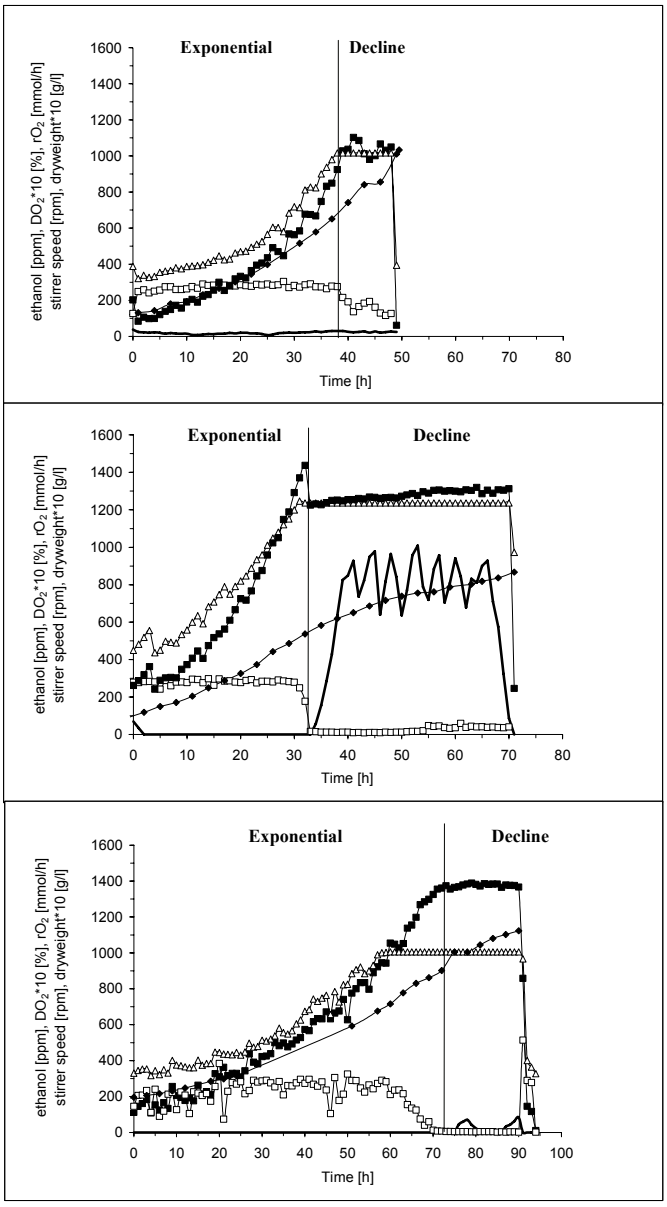

Figure 3.

Feed phases of ethanol fed-batch fermentation profiles with *S. cerevisiae* producing VHH-BC15. Ethanol ppm offgas (▬), DO_2*10 % (–□–), rO_2 mmol/h (–■–), stirrer speed rpm (–△–), dry weight g/l (–◆–). A: Glucose fed-batch fermentation. Exponential feed phase under glucose limitation at a growth rate of 0.06 h^{-1}. Decline phase under glucose limitation and DO_2-concentration of minimal 10%. B: Ethanol fed-batch fermentation. Exponential feed phase under ethanol limitation at a growth rate of 0.06 h^{-1}. Decline phase under ethanol accumulation of approximately 1000 ppm in offgas and a DO_2-concentration close to 0%. C: Ethanol fed-batch fermentation. Exponential phase under ethanol limitation at a growth rate of 0.03 h^{-1}. Decline phase under ethanol limitation and a DO_2-concentration close to 0%

There the glucose fermentation had a maximum productivity of approximately 60 units/kg and decreased during exponential growth. The ethanol fermentations with the same growth rate of 0.06 h^{-1} reached a higher maximum specific productivity of approximately 90 units/kg and did not decrease at the later stages of exponential growth.

To explore the influence of an even lower growth rate on the productivity an ethanol limited fermentation was performed at 0.03 h^{-1} in the exponential phase (figure 3C). Because heterologous protein expression in this experiment was induced in the batch phase the specific productivity is already at maximum at the start of the feed. For the fermentation with 0.03 h^{-1} growth rate this production was maintained until 73h whereas the 0.06 h^{-1} immediately went into the decline phase. This equal maximum production of 90 units/kg indicates that there is no growth rate dependent productivity at this stage of the fermentation when using ethanol (figure 4).

This is further confirmed in table III where the total specific production (Ypx) of the exponential phase is shown. The overall specific productivity (qP) however for the 0.03 h^{-1} ethanol fermentation is the lowest, even below the value for the glucose fermentation.

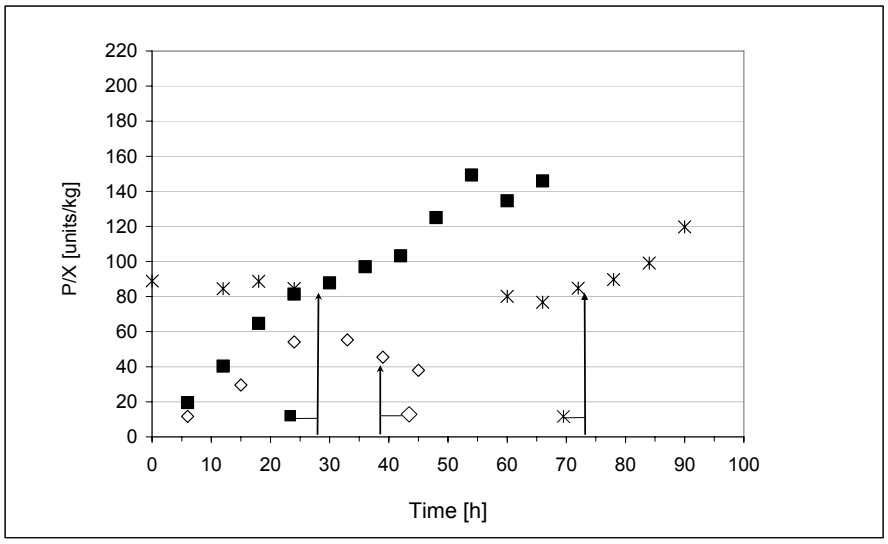

Figure 4.

Specific VHH-BC15 production (P/X) in three different fed-batch fermentations with *S. cerevisiae*. Time was normalised to the start of VHH-BC15 production. Arrows point at the beginning of the decline phase for each fermentation.

Glucose 0.06 experiment (◇), ethanol 0.06-1000 experiment (■), ethanol 0.03 experiment (✳).

Ethanol excess during exponential growth

After investigating the effects of ethanol limitation on VHH-BC15 production, the influence of ethanol accumulation was studied. Therefore several fed-batch fermentations were performed under different non-limiting ethanol concentrations in the exponential phase (figure 5).

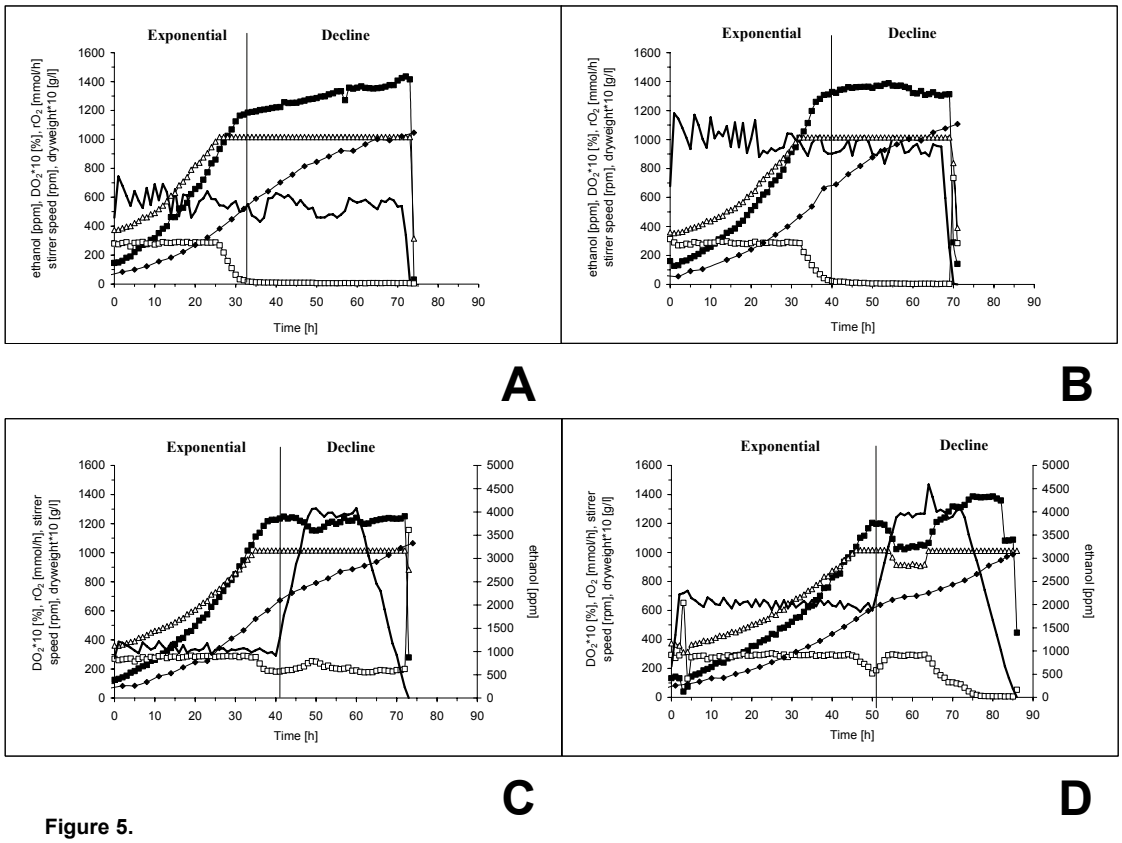

Figure 5.

Feed phases of ethanol fed-batch fermentation profiles with *S. cerevisiae* producing VHH-BC15.
Ethanol ppm offgas (▬), DO_2*10 % (–□–), rO_2 mmol/h (–■–), stirrer speed rpm (–△–), dry weight g/l (–◆–).
A: Ethanol 500-500. Exponential phase with ethanol accumulation of approximately 500 ppm in offgas. Decline phase with same ethanol accumulation and a DO_2-concentration close to 0%. B: Ethanol 1000-1000. Exponential phase with ethanol accumulation of approximately 1000 ppm in offgas. Decline phase with same ethanol accumulation and a DO_2-concentration close to 0%. C: Ethanol 1000-4000. Exponential phase with ethanol accumulation of approximately 1000 ppm in offgas. Decline phase with ethanol accumulation of approximately 4000 ppm and a DO_2-concentration higher than 0%. D: Ethanol 2000-4000 Exponential phase with ethanol accumulation of approximately 2000 ppm in offgas. Decline phase with ethanol accumulation of approximately 4000 ppm in offgas and a DO_2-concentration reducing to 0%.

The loss of ethanol due to evaporation in these experiments was not more than 5%: much less than in the batch fermentations, but still significant. The growth rates in these experiments (figure 6) reduced from the start of the feed from 0.1 h^{-1} to 0.06 h^{-1} and 0.04 h^{-1} for the 2000 ppm ethanol accumulation experiment. So it seems that under these high ethanol concentrations, growth was more strongly inhibited as the culture is subjected to ethanol for a longer period, similar to the observations in the batch experiments.

The specific production of VHH-BC15 in the accumulation experiments is plotted in figure 7. These biphasic graphs show that at the end of the exponential phase the increase in the specific production slowed down to a maximum varying from 148 to 180 units/kg. This is also

visible in table III where the total product yield (Ypx) and the specific productivity (qP) are significantly higher for all accumulation experiments. Unlike the batch fermentations however, there does not seem to be a direct relation between the increase in the ethanol concentration and the increase in specific production (figure 7). But these experiments show that ethanol accumulation in the exponential phase induces a two times higher specific production level than ethanol limited fermentations.

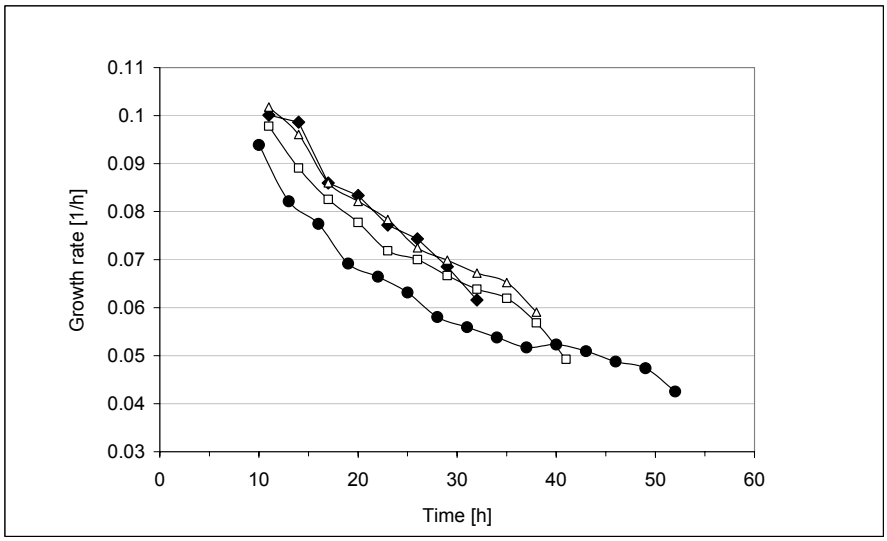

Figure 6.

Growth rates during the exponential phase of fed-batch fermentations under ethanol accumulation with *S. cerevisiae* producing VHH-BC15. Ethanol accumulation levels: 500 ppm (◆), 1000 ppm (□), 1000 ppm (△), 2000 ppm (●). The growth rate for each fermentation was calculated based on rO_2 offgas data. The growth rate of the 2000 ppm ethanol in the broth seems to stronger reduce the growth rate.

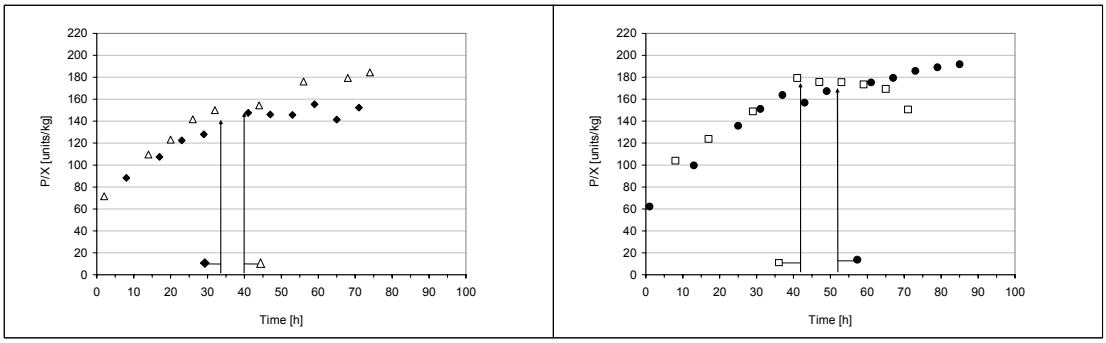

Figure 7.

Specific VHH-BC15 production (P/X) in fed-batch fermentations with *S. cerevisiae* under ethanol accumulation. A: 500-500 experiment (◆), 1000-1000 experiment (△). B: 1000-4000 experiment (□), 2000-4000 experiment (●). The beginning of the decline phase is indicated by the arrows at respectively: 33h, 40h, 42h, 52h. All data are derived from single experiments with errors in dry weight analysis of approximately 10% and product analysis of less than 5%. The profiles are however similar for all experiments, increasing strongly to a maximum level at the moment when the decline phase starts.

Decline phase

In the decline phase of the fed-batch fermentations the oxygen transfer rate is maximal, but nevertheless due to the very high biomass concentration oxygen availability is limiting the growth of the culture, resulting in a declining growth rate (figure 8). The specific production in this phase however does not change significantly in the ethanol accumulation experiments (figure 7). In the limited ethanol experiments (figure 4), the specific production seems to increase to this same maximum level in the decline phase as soon as the ethanol accumulation has started. This also occurs in the experiment with a growth rate of 0.03 h^{-1} where some ethanol accumulation is visible in the fermentation profile. This maximum specific production is thus maintained while growth is declining which demonstrates that there is no effect of growth rate on this maximum specific production.

In table III the overall production yield (Ypx) of the decline phases are shown. Compared to the exponential phase, the decline phase shows a decreased product yield. But these values are significantly higher for the fermentations with accumulation. The relation between the actual ethanol concentration in the broth and the production yield seems to be in favour of low accumulation levels. Because in the high accumulation fermentations (4000 ppm) the yield and productivity were lower than in the 500 and 1000 ppm ones. Furthermore, a small accumulation in the decline phase of the 0.03 h^{-1} experiment already induced a production increase.

Comparing the specific productivities for both exponential and decline phase a clear optimum is not evident. But an ethanol level of 1000 ppm in the exponential and in the decline phase would seem most favourable for a fed-batch process.

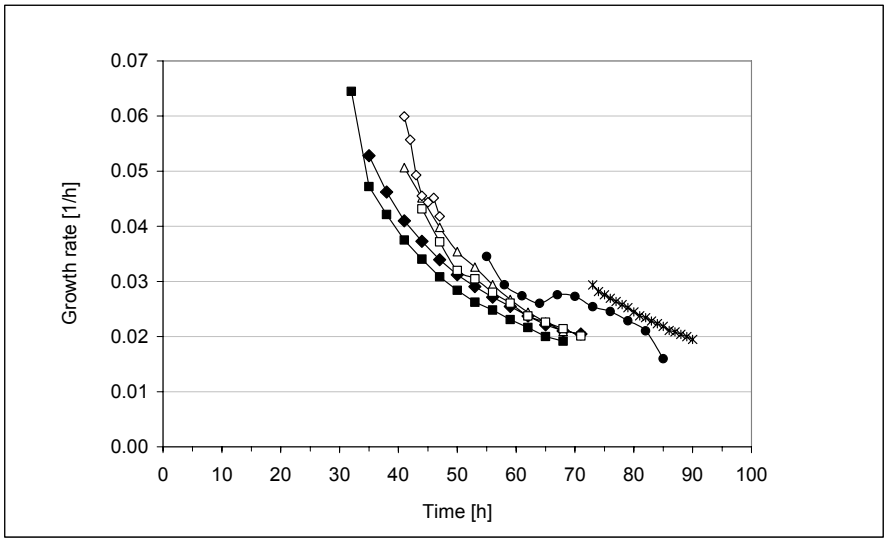

Figure 8.

Growth rates during the decline phase of fed-batch fermentations with *S. cerevisiae* producing VHH-BC15. Experiments: Glucose 0.06 (◇), ethanol 0.06-1000 (■), ethanol 0.03 (✳), 500-500 (◆), 1000-1000 (△), 1000-4000 (□), 2000-4000 (●). The growth rate for each fermentation was calculated based on rO_2 offgas data.

The production limitation in ethanol fermentations

The results of the ethanol fed-batch fermentations showed that in the decline phase the specific production is maximal and remains at that level under limiting conditions and decreasing growth rate. Until production starts to decrease and the system becomes inefficient the fermentation process could thus be further extended. To investigate when this production limiting point arises, increasing amounts of water in the medium were replaced by ethanol. With this method the amount of carbon source can be increased and the free water concentration decreased, without changing the final volume of the fed-batch process. Table IV shows the amounts of water and ethanol in the different experiments. For these experiments the AFP producing strain *S. cerevisiae* CENpk338 was used in ethanol fermentations with 1000 ppm accumulation in both the exponential and decline phase.

Table IV.

Water and ethanol ratios of the total volume in extended ethanol fermentations with *S. cerevisiae* CENpk338. Five fed-batch fermentations were performed with a final weight of 10 kg. Water was taken out of the fermentation medium and replaced by ethanol. Hereby the amount of carbon source increases and the water concentration decreases. This was done to study the limitations in an ethanol fed-batch fermentation.

Fermentation	Total volume	Water	Ethanol
1x ethanol	10 kg	73%	20%
1.2x ethanol	10 kg	69%	24%
1.5x ethanol	10 kg	63%	29%
2.0x ethanol	10 kg	49%	39%
2.4x ethanol	10 kg	44%	47%

Figure 9A shows the oxygen consumption rates (rO_2), indicating the growth of the culture. They show that the rO_2 more rapidly decreased at a time point coinciding with a decrease in the total product in the fermenter (figure 9B). Apparently when growth rapidly drops, heterologous protein in the broth is broken down. The plot of the specific production versus the biomass concentration (figure 9C) shows that these decreases in productivity began at a biomass concentration of 130 g/kg.

To investigate if these effects are related to the water concentration and if production can be restores, several dilutions with water were applied in the cultivation with 2x the standard amount of ethanol. Indeed, at those time points (136h, 140h, 144h), the rO_2 increased and in figure 9B it can be seen that also the total amount of product again increases. The fact that water addition apparently lifts the limitation strongly suggests that the water activity is too low and thus the concentrations of biomass, excreted metabolites and proteases are too high. But also extreme oxygen limitation at these biomass concentrations could induce a change in the

systems productivity. Nevertheless, the results show that the decline phase on ethanol can be extended while remaining highly productive for longer times.

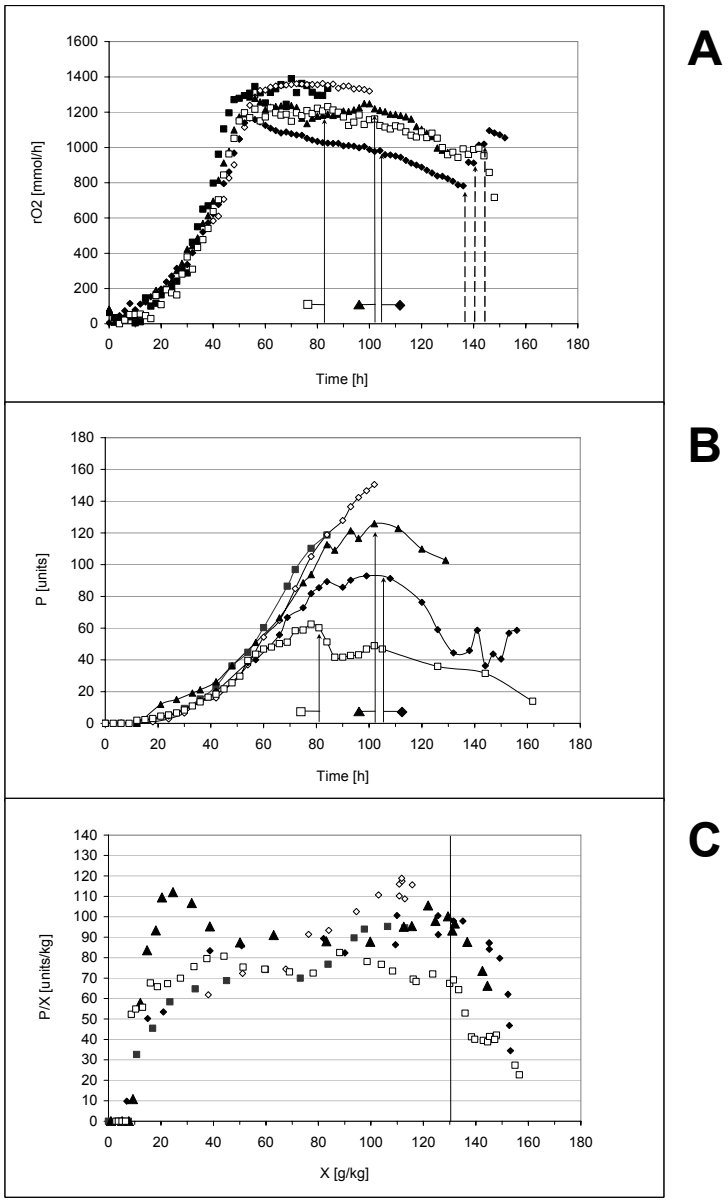

Figure 9.

Ethanol fed-batch fermentations with *S. cerevisiae* CENpk338 producing AFP. To induce strong limitations towards the end of fermentation water in the medium was replaced by ethanol to give 1x the standard amount of ethanol (■), 1.2x ethanol (◇), 1.5xethanol (▲), 2x ethanol (◆) and 2.4x ethanol (□).

A: The oxygen consumption rate (rO_2) during cultivation as indication for growth. At the time points indicated by the arrows the rO_2 significantly decreases. In the experiment with 2x ethanol, water was added to lift the limitations, indicated by the dashed arrows.

B: The total product (P) during the cultivations. The arrows indicate the time points of the rO_2 collapse, coinciding with product breakdown.

C: The specific production (P/X) for the several cultivations plotted versus the biomass concentration in the fermenter. It shows that the productivity collapses around a biomass concentration of 130 g/kg dry weight.

Other heterologous proteins on an optimal ethanol profile

To verify that an ethanol fed-batch process can significantly increase the specific production of different heterologous proteins, several production strains were grown on an exponential glucose protocol and an optimal ethanol fermentation protocol where the ethanol levels after the batch phase were maintained at 1000 ppm in the offgas. The results are shown in table V. All heterologous proteins produced with the exception of AFP, were VHH llama antibody fragments of which the frameworks are highly homologous. Remarkably the VHH production numbers on glucose as sole carbon source vary greatly, although the different VHH proteins are very similar in size and amino acid sequence. Apparently a small change in amino acid sequence can affect correct folding efficiency and therefore secretion values[18].

Table V.

Overall yields for fed-batch fermentations with ethanol (etoh) or glucose (glu) for several different heterologous proteins in *S. cerevisiae* CEN.PK102-3Agal1 producing VHH and CENpk338 producing AFP. The total product, the product yield on biomass and the specific productivity are shown. The VHH products are highly similar in size and amino acid sequence. Still the productivities on glucose vary significantly. When produced in the ethanol protocol the productivity increases for all proteins, especially the low producing ones. The VHH-BC6CA is the same as VHH-BC6 plus an additional cysteine and alanine residue at the C-terminal end. On glucose the secretion is apparently strongly inhibited by this extension while ethanol cultivation increases productivity close to the one without the cysteine extension.

Product	Protocol	t [2] (h)	P_{eof} [3] (units)	Ypx_{ov} [4] (units/kg)	qP_{ov} [5] (units/kg/h)
AFP	glu 0.06 [1]	75	40.4	34.1	0.46
	etoh 0.06-1000 [1]	84	91.8	83.8	1.00
	etoh 1000 ppm	87	106.6	97.7	1.12
VHH-BC15	glu 0.06 [1]	66	34.1	30.7	0.46
	etoh 0.06-1000 [1]	84	155.9	145.9	1.74
	etoh 1000 ppm	81	206.6	178.9	2.21
VHH-BC6	glu 0.06	57	24.8	26.6	0.47
	etoh 1000 ppm	87	58.5	48.7	0.56
VHH-BC6CA	glu 0.06	60	4.6	4.9	0.08
	etoh 1000 ppm	92	39.0	40.4	0.44
VHH-BCF	glu 0.06	63	42.1	46.1	0.73
	etoh 1000 ppm	87	143.2	125.4	1.44
VHH-BC5	glu 0.06 [6]	66	83.7	95.3	1.44
	etoh 1000 ppm	105 [7]	139.8	128.1	1.22

1) From: Chapter 2, this thesis[5]
2) Total fermentation time
3) Peof: Total product at end of fermentation
4) Ypx_{ov} : Overall yield product per biomass. Calculated by P_{tot}/X_{tot}
5) qP_{ov}: Overall specific productivity. Calculated by $P_{tot}/(X_{tot}*t)$
6) Glucose fermentation performed at 30°C in stead of 21°C
7) 27 hours of lag time in fermentation: decreased qP

With the use of the ethanol protocol, the specific production of all the fermentations increased dramatically (table V). The 1000 ppm ethanol protocol gave a higher increase than the protocols without the ethanol accumulation, as seen for VHH-BC15 and AFP. Also the overall specific productivity (qP) increased. The effect of ethanol in increasing the production apparently applies to many heterologous peptides but the degree of increase is different for each protein. Some VHH fragments show a clear difference on ethanol (VHH-BC15 and VHH-BCF) and for others it is smaller (VHH-BC5 and VHH-BC6).
Remarkably though is the difference between the production of VHH-BC6 and VHH-BC6CA. The latter one is the same VHH but with a C-terminal cysteine and alanine. This unpaired terminal cysteine severely affects protein folding in the ER by formation of disulphide bridges. The productivity therefore is extremely low in glucose cultivation, compared to the original protein. However, when cultivated on ethanol the production increases dramatically to the same level as VHH-BC6. This strongly suggests that the effect of ethanol on the increased production of heterologous proteins is related to protein folding and redox processes. Apparently ethanol changes the cells state to such an extent that impaired secretion can be overcome and production can be greatly increased.

Discussion

For designing an optimal industrial fed-batch fermentation with ethanol as sole carbon source, the key characteristics of growth and heterologous peptide production of *S. cerevisiae* were determined.
Batch incubations showed that high amounts of ethanol decrease the growth rate but strongly increase specific production. Still growth rates are higher than the optimum of 0.06 h^{-1} for glucose fed-batch cultivation. Too high ethanol concentrations lead to enhanced evaporation of the carbon source and decreased growth rate resulting in extended cultivation times.
Fed-batch fermentations were used to study these events in more detail. Ethanol limited cultivation at different low growth rates showed a plateau value in the specific production. When growth rates higher than 0.06 h^{-1} are applied ethanol accumulation occurs as the feed rate exceeds the ethanol consumption rate. Ethanol accumulation however in all cultivations induced a strong increase in production. Also in the decline phase this was seen and moreover the specific production in this stage was found to remain at a maximum level regardless of growth rate, dissolved oxygen level and also the height of ethanol accumulation. However under too high ethanol concentrations the overall specific productivity of the fermenter decreases due to growth inhibition.
Based on these findings an optimised fed-batch process would comprise a batch phase with excess glucose as carbon source for primary biomass production and a smooth transition to ethanol feed due to the Crabtree effect[19]. An ethanol accumulation of 1000 ppm in the offgas (approximately 1%v/v in the broth) should then be maintained throughout the whole feed

phase. Employing such a feed strategy has resulted in 4.8x higher specific productivity than a standard glucose fed-batch fermentation.

So far this ethanol fed-batch fermentation process has shown no production limitation. Under oxygen limitation and a declining growth rate the culture still produces at a maximum rate and the fermentation can therefore be continued until the fermenter has reached its ultimate volume. A glucose fed-batch fermentation would have been terminated as the productivity will decrease under the same limiting conditions. To fully test the scope of the ethanol process it was proven that the high productivity of the fermentation could not be maintained indefinitely. Upon exchanging water for ethanol the productivity and growth collapsed when the biomass concentrations reached 130 g/kg. Under these high cell density conditions many effects take place that can induce this. It is unlikely that viscosity is the reason for this as yeast suspensions at these biomass concentrations are reported to still behave as Newtonian liquids[20,21]. Impaired oxygen transfer in the medium or anaerobic micro environments can contribute to this effect although mixing times are low in the 10 L system[22].

Another reason for the decreased production and metabolism could be the high concentrations of metabolic by-products, CO_2 and proteases, which are evident in any high cell density cultivation[23]. In general however, it is most likely that the water activity becomes too low for *S. cerevisiae* to stay metabolically active. Hallsworth[24] described the minimum water activity (a_w) to be 0.94, beneath which the growth of most brewing strains is strongly inhibited. This a_w value is already reached in a 10%w/v ethanol solution and although the ethanol concentration in the broth does not increase above 1%v/v, the water concentration is extremely low (table IV). It is therefore evident that the final limitation on the heterologous protein production is the fermenter volume or the water concentration in the broth. This opens the possibility to use fermentation techniques such as repeated fed-batches to further increase the volumetric productivity[25,26].

The optimised protocol with the 1000 ppm ethanol was finally tested on several other heterologous proteins produced in this *S. cerevisiae* system. It appeared that although the proteins produced are very homologous, large differences in production levels were observed. On the ethanol protocol this production increased in all cases and in some cases dramatically. As to the exact reason for the increased protein production with ethanol as carbon source, no direct evidence was found. But due to the fact that ethanol increases the production of a protein with a C-terminal cysteine it is very likely that ethanol induced stress directly affects the protein processing and secretion efficiency. But also metabolic and energetic changes are evident in these fermentations. DNA microarray analysis could elucidate the true effect and will be described in future work.

Irrespective of the true mechanism behind the ethanol effect, the ethanol based fed-batch fermentation can be considered as a general applicable and highly productive system for heterologous protein production[27].

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CHAPTER 4

Genomic analysis of heterologous protein producing *Saccharomyces cerevisiae* in ethanol fed-batch fermentations

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Abstract

The production of heterologous proteins by *S. cerevisiae* is mostly performed in fed-batch fermentations with glucose as carbon source. Recently we found that the fermentation on ethanol in stead of glucose significantly increased the productivity of heterologous proteins.
This cultivation and the production of large amounts of heterologous proteins have a strong effect on the state of the yeast cell. Although much knowledge is generated on *S. cerevisiae*, even in the presence of ethanol, this is often limited to defined laboratory conditions in the absence of the heterologous production burden. Therefore we performed DNA microarrays on glucose and ethanol fed-batch cultivations to investigate the metabolic and physiological state of the yeast under ethanol conditions and low growth rate.
The genome wide DNA analyses confirmed that many processes like the central carbon metabolism were dramatically changed. Nitrogen and fatty acid metabolism play an important role by directing intermediates to the central metabolism to compensate for the carbon and energy shortage. A large up regulation of the catabolism of proteins was witnessed, however the ERAD response, normally up regulated in *S. cerevisiae* producing heterologous proteins was not up regulated. We also observed that in spite that the concentration of glucose in a fed-batch phase is practically zero, the genes related to galactose metabolism were strongly up regulated. Also a very strong redox and oxidative stress effect was found.
The two main up regulated effects in the cellular response to ethanol seem to be catabolic and transport. The relation of this response with increased protein production on ethanol will be further studied following this work.

Introduction

In spite of a history of more than 25 years, the industrial production of heterologous proteins by *Saccharomyces cerevisiae* is still under investigation for further optimisation, because each heterologous protein can bring about its own difficulties. Most commonly improvements are performed to reach higher production yields by strain mutagenesis and fermentation development. For the production of Camelid antibodies (VHH) by *S. cerevisiae* a fed-batch protocol was developed that uses ethanol in stead of glucose as sole carbon source[1] with galactose as inducer of the transcription of the heterologous gene. Under these conditions the specific VHH productivity of the yeast was increased up to a factor 5 compared to similar glucose fed-batch fermentations. It was noticed that under the stress conditions present in the final phase of the ethanol fermentation, in which the growth rate decreased, the specific production remained the same. This implied that under these conditions a relative large proportion of the nutrients and energy was used for the production of the heterologous product. It is important to understand the cell physiology under these ethanol conditions in

order to elucidate the mechanism for enhanced production on ethanol and to improve this process even further.

The effects of ethanol on *S. cerevisiae* have been studied extensively. It is a less efficient carbon and energy source which yields lower amounts of biomass[2] and it decreases the growth rate[1,3]. In addition, it has been described to be toxic as it acts on the fluidity of the plasma membrane[4] which affects permeability and transport[5] and it induces a strong stress response[6]. Recently many studies on the effect of ethanol have been performed using DNA-microarrays, which enormously increased the amount of data on this subject. However, these studies were all performed under different conditions. Some of these experiments were performed after fermentative glucose cultivation by addition of pulses of ethanol[4,7], while others studied ethanol metabolism after a diauxic shift[8] or under brewing conditions[9]. Further studies analysed the stress response in stationary shake flask cultures[10]. Daran-Lapujade *et al.*[11] analysed the ethanol metabolism compared to glucose in continuous cultures, which describes more accurately the response of ethanol metabolism in steady state cells.

However, none of these analyses were carried out under conditions comparable with industrial fed-batch fermentations or under heterologous protein production[12]. Therefore we performed a set of DNA-microarray analyses to dissect the role of ethanol and low growth rate on VHH production in such processes.

The results described here are in line with the data available for growth on ethanol and show the extreme complexity in cell response, however we also found additional influences on metabolism and transport that could give important leads for further process improvement.

Materials and methods

Strain

Saccharomyces cerevisiae strain CEN.PK102-3Agal1 (*gal1:URA3*, *leu2*, *ura3*) carried a multi copy integration vector, integrated at the rDNA locus[13], containing the nucleotide sequence encoding the Llama variable domain VHH-BC15 gene linked to a synthetic signal sequence Hmss1 under control of the *GAL7* promoter and *leu2d* selectable marker to maintain a high copy number. The described strain secreted VHH-BC15 into the fermentation medium.

Fed-batch fermentations

The rich medium used for preparation of the inoculum and in the fed-batch fermentations is described in van de Laar *et al.*[1]. The fermentations were performed in standard fermenters with a 10 L working volume according to same protocol. The batch phase (30°C, 2 L/min air, DO_2 minimum 30%, pH 5.0) was started by inoculation with 500 ml full-grown YPD (1% yeast extract, 2% peptone, 2% glucose) culture. The feed was automatically started when the ethanol concentration decreased below 300 ppm. In the feed phase (21°C, 6 L/min air) the

exponential feed rate was applied to maintain a growth rate of 0.06 h^{-1}. In the glucose fermentation, the feed rate was set to linear when the DO_2 level decreased below 15%. In the ethanol fermentation the DO_2 decreased to 0% and subsequently accumulating ethanol increased to a maximum of 1500 ppm in the off-gas. Then, to maintain the ethanol level between 800 and 1500 ppm a pulsed feed profile was applied.

Sampling, RNA isolation and microarray

Samples for microarray analysis were taken at two different time points from a glucose (G1 and G4) and an ethanol (E1 and E4) fed-batch fermentation. The samples were taken directly from the fermenter and immediately frozen in liquid nitrogen. The time points of each sample correspond to the exponential phase after approximately 26 hours (G1 and E1) and decline phase after approximately 66 hours (G4 and E4). An overview of the samples and the characteristics is given in table I.

For RNA isolation, samples were thawed on ice and approximately 100 mg cells were resuspended in 12 ml AE-buffer (50 mM NaAcetate pH 5.2, 10 mM EDTA). RNA extraction was performed by the method of Llinas[14]. The purity and integrity of the samples were verified by an RNA labchip 2100 bioanalyzer from Agilent technologies. Before hybridisation the 4 regular samples were treated and their quality verified according to Affymetrix protocols[15]. DNA microarrays were performed by hybridisation of the samples to the Affymetrix GeneChip Yeast Genome S98 Array according to Affymetrix protocols. The arrays were scanned by a GeneArray Scanner system and data were extracted with microarray Suite 5.0.

Table I.

Key characteristics of the samples for DNA microarray analysis. Two fed-batch fermentations with *S. cerevisiae* CEN.PK102-3Agal1 producing VHH-BC15 were performed, one on glucose and one with ethanol as sole carbon source. Samples were taken during early cultivation (exponential growth) phase and in the final phase (decline) of cultivation under decreasing growth rate.

	Glucose		Ethanol	
	G1	G4	E1	E4
Fermentation time	24 h	65 h	26 h	67 h
Biomass	18 g/kg	101 g/kg	22 g/kg	78 g/kg
Growth rate	0.06 h^{-1}	0.03 h^{-1}	0.06 h^{-1}	0.03 h^{-1}
DO_2	30%	15%	30%	0%
Fermentation phase	exponential	decline	exponential	decline
Carbon source	limited	limited	limited	1%v/v accumulation

Data processing and criteria for up- or down regulation of genes

Raw data were analysed in dChip as described by Zakrzewska *et al.*[16] for assessing standard errors and normalising the data. The 4 datasets were together analysed against the G1

sample. The obtained expression levels were converted into an MS excel format and expression ratios based on ^{2}Log values were calculated. The following response criteria for the expression ratios were used: high down regulation (ratio ≤ -1), down regulation (-1 < ratio ≤ - 0.2), no response (-0.2 < ratio < 0.2), up regulation (0.2 ≤ ratio < 1), high up regulation (ratio ≥ 1).

Function classes for the identified genes were determined based on Gene Ontology annotations[17] in the *Saccharomyces* genome database (SGD)[18] and GenMAPP[19], a gene map annotator and profiler program. Only open reading frames with a high up regulation or a high down regulation were selected for further analysis. Subsequently the high up- or down regulation of a complete GO functional group was determined by analysing the significance by MAPPfinder[20] for a z-score higher than 2.5 and a p-value below 0.01. Further analysis of a GO group was done in GenMAPP based on the Sc-Std_20040411 gene database of *S. cerevisiae*. By this analysis only defined GO function groups are analysed when they are significantly high up- or down regulated. As gene ontology is a hierarchical clustered database, many parent-child relationships are possible. In the case that both parent and child group contain the same genes, the parent group was eliminated form the selection table.

Ammonia and Phosphate analysis

The ammonia and phosphate concentration in cell free fermentation samples was determined by spectrophotometrical determination on a Cobas Mira autoanalyser (Roche). The solutions used for the ammonia assay were 0.1 M KH_2PO_4 (Merck) reagent, 100 g/l sodium hypochlorite (Merck) and 1.6 M NaOH (Merck) reagent, 0.85 mM Na-nitroprusside (Merck) and 0.53 M phenol (Merck) reagent. For the phosphate assay the following solutions were used: 7.82 g/l polyvinylpyrrolidon and 11% sulphuric acid reagent, 34 mM ammonium heptamolybdate reagent and 5 g/l ascorbic acid reagent. The reactions were incubated at room temperature and the absorption increase was measured at 600 nm for ammonia and 340 nm for phosphate. Concentrations were then calculated based on a calibration curves.

Results

Sample characteristics

A summary of the data of the fed-batch fermentative production of the variable domain of Camelid heavy chain antibody[1] by *Saccharomyces cerevisiae* with glucose and ethanol as main carbon and energy sources is given in figure 1. As the objective of this study was to understand why the production of heterologous proteins by *S. cerevisiae* in fed-batch ethanol processes was higher than in similar fed-batch processes on glucose, we compared three different pair wise conditions (G4, E1 and E4) with G1. The latter condition is most related to the conditions described in literature and therefore serves as reference point. In fact these

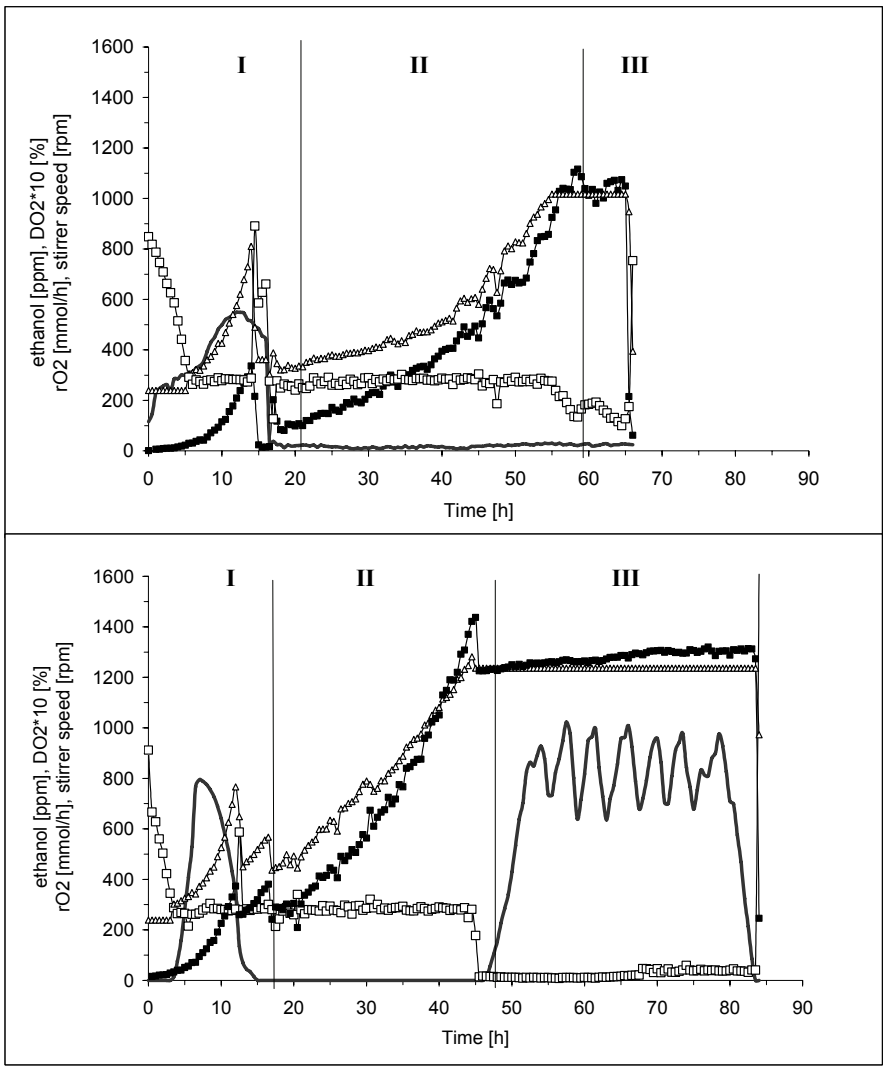

Figure 1.

Fermentation profiles of glucose (A) and ethanol (B) fed-batch fermentations with *S. cerevisiae* producing VHH-BC15.

Ethanol ppm offgas (▬), DO_2*10 % (–□–), rO_2 mmol/h (–■–), Stirrer speed rpm (–△–).

I: Batch phase on glucose. **II**: exponential feed phase on glucose or ethanol. **III**: Decline phase with linear glucose feed and decreased DO_2 or ethanol accumulation and oxygen limitation.

After the batch phase the limiting exponential feed is started, resulting in a carbon source concentration of approximately zero in the broth. As the oxygen consumption increases, so does the stirrer speed until its maximum value. In the glucose fermentation the feed rate is then set to linear to maintain a DO_2 above 10%. In the ethanol fermentation the DO_2 is allowed to decrease to 0% and ethanol accumulates in the broth to approximately 1000 ppm in the offgas, which is approximately 1%v/v in the broth.

van de Laar *et al*, Chapter 2, this thesis[1]

comparisons analyse the following parameters: reduction in growth rate (G4 vs G1), difference in main carbon source and mild ethanol stress (E1 vs G1), externally induced stresses, such as a moderate concentration of ethanol and oxygen depletion in combination with ethanol as main carbon source and low growth rate (E4 vs G1). The G1 and E1 samples were taken from a carbon source limited pseudo steady state, 15 hours after the start of the

exponential feed at a growth rate of 0.06 h^{-1}. The E4 sample was taken in the decline phase, 22 hours after the start of ethanol accumulation, where oxygen was limiting the culture to a growth rate of 0.03 h^{-1}. The G4 sample was taken also in the decline phase at a growth rate of 0.03 h^{-1}, 10 hours after the maximum rO_2 was reached. The DO_2 was maintained above 10% to maintain a glucose limited culture.

Functional analysis of high up and down regulated genes

High responsive genes (ratios ≤ -1 and ≥1) were analysed according to their gene ontology annotation for a z-score above 2.5 and a probability p-value lower than 0.01[20]. Of the three main subclasses of GO, only biological processes and cellular components were further analysed. The groups complying with these criteria were then classified in categories of cellular functionality. The results are shown in table II and III (Page 86 and 87) for respectively high up regulation and high down regulation. The complete data set of this study and the list of GO annotation results classified to their relevance are available for download at http://www.bac.nl/news/Data-PhD-thesis/20.

Comparison of E1 with G1

1. Carbon metabolism

Figures 2 and 3 illustrate clearly that various parts of the carbon metabolism are up regulated by cultivation on limited amounts of ethanol in stead of glucose, while other parts are down regulated comparable to results described in the literature[4,8,11]. Ethanol passively diffuses into the cell and is metabolised via acetaldehyde and acetate into acetyl-CoA, yielding NADH and NADPH. Acetyl-CoA is subsequently metabolised in the tricarboxylic acid pathway (TCA), of which nearly all genes encoding TCA enzymes are up regulated (figure 3). The glyoxylate cycle and gluconeogenesis direct the carbon flux towards phosphoenol pyruvate and upwards to cell wall components and protecting or energy storage sugars. Genes encoding enzymes of these processes are strongly up regulated (figure 2).

On limited ethanol the pentose phosphate pathway (PPP) is down regulated compared to glucose cultivation, most likely because NADPH, which is necessary for assimilatory pathways and in yeast not convertible from NADH[21], is now generated by acetaldehyde and isocitrate dehydrogenases[11]. The biosynthesis of glycerol is also down regulated while the glycerol catabolism is up regulated by increased expression of the *GUT1* and *GUT2* genes, which are under glucose repression. As can be expected, glucose transportation and the first steps in glycolysis are strongly down regulated during growth on ethanol.

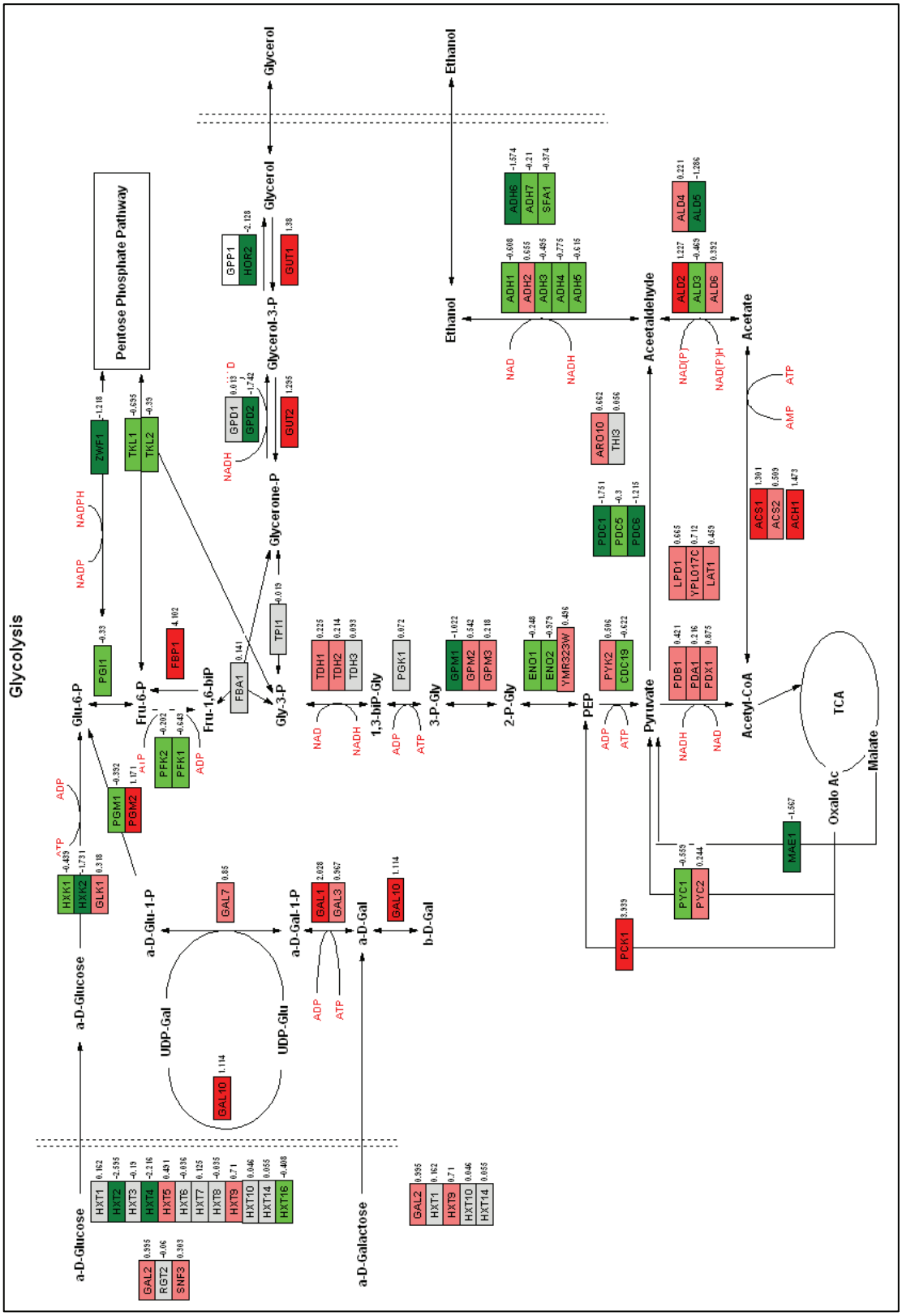

Figure 2.

E1vsG1 expression ratios of genes involved in glycolysis and related pathways.

Comparison of ethanol (E1) and glucose cultivation (G1) during the exponential growth phase in fed-batch fermentations with *S. cerevisiae* producing VHH-BC15.High up regulation on ethanol in dark red, up regulation in light red. High down regulation on ethanol in dark green, down regulation in light green. Grey: no criteria met, white: no data found. The pathway model is based on data from SGD[18] and KEGG[60].

Table II.

High up regulated GO functions in the three different expression comparisons. GO groups are selected when they are generally high up regulated (ratios≥1) according to a z-score above 2.5 and a probability p-value lower than 0.01. Subsequently they are allocated to function groups according to the first column and further discussed in the text.

	E1vsG1 HIGH UP GO term	E4vsG1 HIGH UP GO term	G4vsG1 HIGH UP GO term
Metabolism			
Metabolism	carbohydrate metabolism carboxylic acid metabolism alcohol metabolism	carbohydrate metabolism carboxylic acid metabolism alcohol metabolism	
Glucose			hexose transport alpha-glucoside transport
Galactose		galactose metabolism	
Gluconeogenesis	gluconeogenesis monosaccharide metabolism	gluconeogenesis alcohol biosynthesis	
TCA	TCA intermediate metabolism propionate metabolism	TCA intermediate metabolism propionate metabolism	
Glyxoylate	glyoxylate cycle	glyoxylate cycle	
Trehalose and glycogen	energy reserve metabolism glycogen metabolism		
Aldehyde metabolism	aldehyde metabolism	aldehyde metabolism	aldehyde metabolism
Peroxysome and fatty acid metabolism			
Carnitine	carnitine metabolism	carnitine metabolism	
Peroxysome	peroxisome peroxisomal matrix integral to peroxisomal membrane protein-peroxisome targeting		
Fatty acid metabolism	lipid metabolism fatty acid metabolism fatty acid oxidation fatty acid beta-oxidation		
Redox and energy			
Energy pathways	energy derivation by oxidation of organic compounds		
NADH	NADH regeneration		
Formate	formate catabolism one-carbon compound catabolism		formate catabolism one-carbon compound catabolism
Protein metabolism and transport			
Autophagy Vacuolar	autophagy protein-vacuolar targeting		
Proteasome			
	ubiquitin cycle protein deubiquitination		
Amino acids, co-factors, ions, nucleotides			
Transport	multidrug transport	transport ion transport anion transport sulfate transport iron-siderochrome transport vitamin/cofactor transport carboxylic acid transport amino acid transport amine/polyamine transport	
Copper iron			copper ion import copper ion transport
Amino acid metabolism		amino acid and derivative metabolism amino acid metabolism amine metabolism one-carbon compound metabolism sulfur amino acid metabolism	
Sulphur related		sulfur metabolism sulfate assimilation	
Vitamins Biotin	water-soluble vitamin metabolism biotin biosynthesis	vitamin metabolism water-soluble vitamin metabolism water-soluble vitamin biosynthesis biotin biosynthesis	vitamin metabolism water-soluble vitamin metabolism water-soluble vitamin biosynthesis biotin metabolism
Pyridoxine Thiamine		pyridoxine metabolism thiamin metabolism thiamin biosynthesis	
Cell cycle			
Sporulation	sporulation prospore membrane spore wall assembly		
Mating	cytokinesis\, completion of separation	cytogamy conjugation with cellular fusion cellular morphogenesis during conjugation	reproduction conjugation with cellular fusion agglutination during conjugation with cellular fusion cytokinesis\, completion of separation
Pheromones		response to abiotic stimulus response to chemical substance response to pheromone during conjugation with cellular fusion	response to chemical substance response to pheromone response to pheromone during conjugation with cellular fusion
Cell wall Cell structure		cell wall (sensu Fungi) membrane	cell wall (sensu Fungi) plasma membrane
Signalling and others			
Others	protein phosphatase type 1 complex		

Table III. High down regulated GO functions in the three different expression comparisons.

	E1vsG1 HIGH DOWN GO term	E4vsG1 HIGH DOWN GO term	G4vsG1 HIGH DOWN GO term
Metabolism			
Metabolism	metabolism carboxylic acid metabolism		
Glucose		carbohydrate transport hexose transport glucose transport monosaccharide metabolism hexose metabolism fructose metabolism alcohol metabolism	
Glycerol		glycerol metabolism glycerol biosynthesis	
Protein metabolism and transport			
	protein biosynthesis		
Amino acids, co-factors, ions, nucleotides			
Transport		organic anion transport	
Amino acid metabolism	amino acid and derivative metabolism amino acid metabolism amino acid biosynthesis amine metabolism amine biosynthesis histidine biosynthesis lysine biosynthesis aspartate family amino acid metabolism aspartate family amino acid biosynthesis methionine metabolism sulfur amino acid metabolism peptidyl-arginine modification peptidyl-diphthamide biosynthesis peptidyl-amino acid modification	amine catabolism	amino acid and derivative metabolism amino acid metabolism amino acid catabolism D-amino acid catabolism amine biosynthesis amino acid biosynthesis amine metabolism aspartate family amino acid metabolism aspartate family amino acid biosynthesis arginine catabolism to ornithine glutamine family amino acid catabolism histidine biosynthesis lysine biosynthesis\, aminoadipic pathway
Sulphur related	sulfur metabolism sulfate assimilation		
Nucleotides	nucleic acid metabolism ribonucleoside metabolism nucleoside monophosphate metabolism pyrimidine nucleotide metabolism pyrimidine base biosynthesis 'de novo' pyrimidine base biosynthesis purine salvage pyrimidine salvage	nucleic acid metabolism	
Cell cycle			
Budding	bud site selection		
Cell structure	cell intracellular cell organization and biogenesis cytoplasm organization and biogenesis cytosol nucleus physiological process biosynthesis macromolecule biosynthesis	cell cell organization and biogenesis cytoplasm organization and biogenesis nucleus	
DNA, transcription, translation			
Transcription	transcription from Pol I promoter transcription from Pol III promoter DNA-directed RNA polymerase I complex DNA-directed RNA polymerase II\, core DNA-directed RNA polymerase III complex RNA processing RNA modification RNA methylation RNA metabolism	transcription from Pol I promoter transcription from Pol III promoter RNA polymerase complex DNA-directed RNA polymerase I complex DNA-directed RNA polymerase III complex RNA processing RNA modification RNA methylation RNA metabolism	telomerase-dependent telomere maintenance
Nucleolus	nucleolus small nucleolar ribonucleoprotein complex ribonucleoprotein complex ribosome-nucleus export	nucleolus small nucleolar ribonucleoprotein complex small nuclear ribonucleoprotein complex ribonucleoprotein complex ribosome-nucleus export	
rRNA	rRNA processing rRNA modification processing of 27S pre-rRNA processing of 20S pre-rRNA 35S primary transcript processing	rRNA processing rRNA modification processing of 20S pre-rRNA 35S primary transcript processing	processing of 20S pre-rRNA
Ribosome	ribosome large ribosomal subunit small ribosomal subunit ribosome biogenesis and assembly ribosome biogenesis ribosome assembly ribosomal subunit assembly ribosomal large subunit biogenesis ribosomal large subunit assembly ribosomal small subunit assembly cytosolic ribosome (sensu Eukarya) cytosolic large ribosomal subunit (Eukarya) cytosolic small ribosomal subunit (Eukarya) eukaryotic 48S initiation complex eukaryotic 43S preinitiation complex	ribosome biogenesis and assembly ribosome biogenesis ribosome assembly ribosomal subunit assembly ribosomal large subunit assembly	ribosomal small subunit biogenesis
tRNA	tRNA metabolism tRNA modification		
Translatie	regulation of translational fidelity translation		
Signalling and others			
Others		response to dessication	

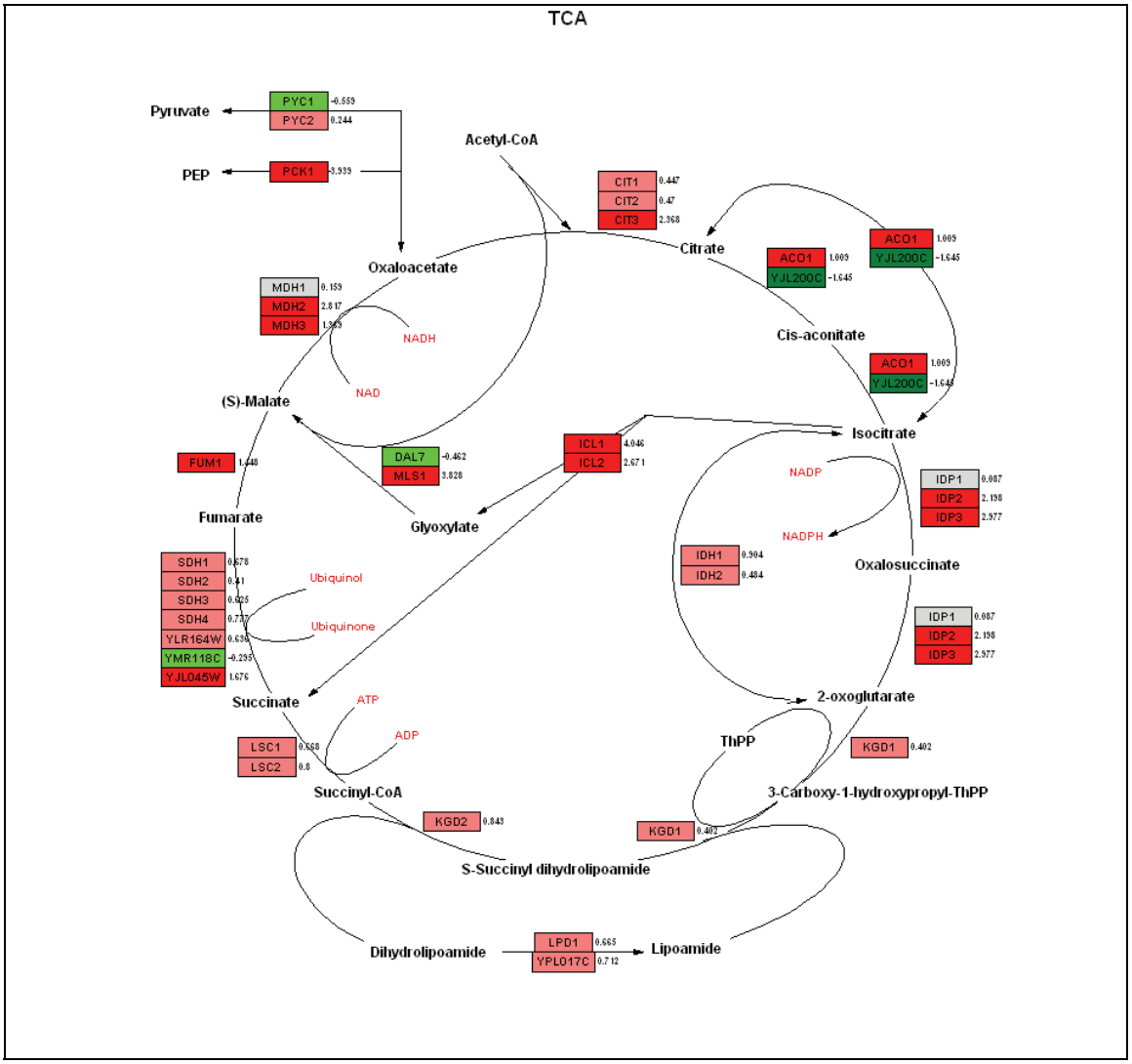

Figure 3.

E1vsG1 expression ratios of genes involved in citric acid cycle (TCA).

Comparison of ethanol (E1) and glucose cultivation (G1) during the exponential growth phase in fed-batch fermentations with *S. cerevisiae* producing VHH-BC15. High up regulation on ethanol in dark red, up regulation in light red. High down regulation on ethanol in dark green, down regulation in light green. Grey: no criteria met, white: no data found. The TCA model is based on data from SGD[18] and KEGG[60]. In general the whole pathway is strongly up regulated on ethanol.

Galactose is available in the medium for induction of the heterologous protein production as the gene encoding the heterologous product is under control of the *GAL7* promoter. To circumvent galactose consumption and consequently the reduction in the level of the inducer, the host strain carries an inactive *GAL1* gene. The induction of genes of the galactose metabolism (figure 4) is much stronger under ethanol than under glucose growth conditions, with the notable exception of *GAL4*, which encodes the central transcription factor of the galactose metabolism[22]. The biosynthesis of glycogen and trehalose are strongly up regulated on ethanol (figure 5) as has been found in other studies[4,7]. In addition to generating storage of carbon and energy, trehalose has been described to have a chaperone function in response

to heat shock and stress[4,23] but the pathway could also be involved in the cell cycle at low growth rate[24].

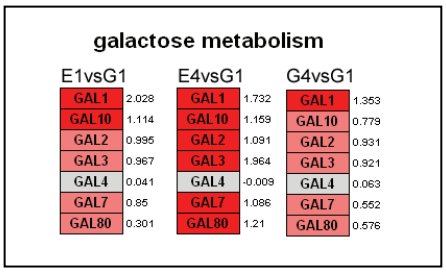

Figure 4.

Expression ratios of genes in the GO groups of "galactose metabolism" compared to standard glucose cultivation (G1). High up regulation in dark red, up regulation in light red. Grey: no criteria met. All genes are high up regulated on ethanol, but also under lower growth rate (E4 and G4), even under glucose growth.

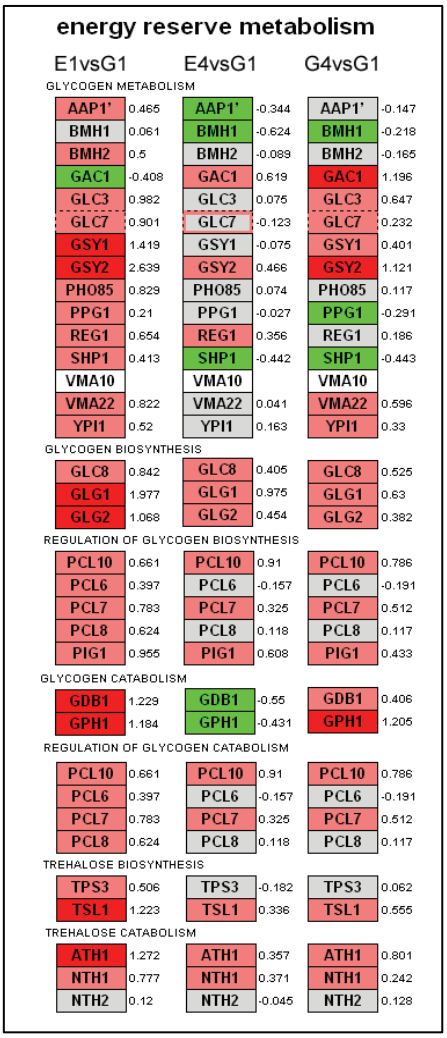

Figure 5.

Expression ratios of genes in the GO group of "energy reserve metabolism" compared to standard glucose cultivation (G1). High up regulation in dark red, up regulation in light red. High down regulation in dark green, down regulation in light green. Grey: no criteria met. White: no data found. In ethanol (E1) cultivation the reserve metabolism is generally up regulated. Under low growth rate (G4) still several genes are up regulated compared to standard glucose growth.

In table II a strong up regulation is shown for the aldehyde metabolism. The extracellular ethanol concentration is approximately 1%v/v and the passive diffusion will also increase the intracellular concentration[3], which will be converted into acetaldehyde. Acetaldehyde has been described to induce a strong STRE-regulated stress response[25] but also induce expression of multi drug transport and sulphur metabolism[26]. Closer scrutiny of this up regulated GO-group shows that 7 of the 10 open reading frames code for aryl-alcohol dehydrogenases (AAD) genes. However not all produce active AAD and several of this group seem to be redundant[27]. Furthermore the exact function of the AAD-genes is unknown,

although their activity is thought to be related to amino acid catabolism[28] they are responsive to oxidative stress which could explain the high up regulation in also the E4 and G4 samples. Apparently this is not purely attributable to growth on ethanol.

2. Peroxisomal activity

It has been described in literature that in yeast growing on rich and complex media only a few and very small peroxisomes are present, but when Candida and Hansenula strains grow on compounds such as ethanol these peroxisomes increase in size and number[29,30]. For *Saccharomyces cerevisiae* so far this has not been clearly shown[31]. It has also been described that production of heterologous proteins may induce proliferation of the ER membranes[32,33] and as mentioned above, the outer membrane is affected by ethanol directly. These observations indicate that a higher turn over of phospholipids in *S. cerevisiae* grown on ethanol and producing a heterologous protein is likely. Indeed, when cultivated on ethanol the GO-groups related to the peroxisome and genes encoding enzymes of the fatty acid oxidation and carnitine transport are strongly up regulated (table II, figure 6). Also phosphatidylcholine biosynthesis is up regulated, which indicates membrane proliferation.

The beta-oxidation of fatty acids yields acetyl-CoA in the peroxisomes that can be used for replenishing the TCA cycle. However it can only be transported over the membranes into the mitochondria by two mechanisms. Via the peroxisomal glyoxylate cycle where acetyl-CoA and oxaloacetate are converted to citrate by Cit2p and subsequently converted to succinate either in the peroxisome or cytosol which is then transported through a succinate carrier (Sfc1p) into the mitochondrion[34].

The other acetyl-CoA transport pathway is through carnitine acetyl transferase[35]. Although *S. cerevisiae* is incapable of synthesising carnitine[36] it can be transported from the yeast extract in the rich medium, where it is present in trace amounts, by the high-affinity glutamine permease Agp2p. The acetyl-CoA is then transferred to and from carnitine by the transferases Cat2p in the peroxisome and in the mitochondrial membrane by Yat1p and Yat2p. Also *CRC1* encoding the mitochondrial carnitine translocase is concerned in this process[36]. In figure 6 it shows that all genes related to this acetyl-CoA transport are strongly up regulated on ethanol, compared to glucose cultivation, which supports the importance of fatty acid beta oxidation in ethanol metabolism.

On growth on ethanol, the genes involved in the mitochondrial (*ACH1*) and peroxisomal (*TES1*) CoA-synthesis are highly up regulated. So the heavy burden on the citric acid cycle for energy, gluconeogenic routes and the extraction of intermediates is countered by acetyl-CoA replenishment through ethanol degradation, fatty acid oxidation and the glyoxylate pathway[37]. Side products of peroxisomal activity are the aldehydes and peroxides which induce reactive oxygen species (ROS) and a strong oxidative stress response[38,39]. This is illustrated by the high up regulation of the peroxisomal catalase *CTA1*. The consequences of the up regulation of genes via the oxidative stress response will be discussed below.

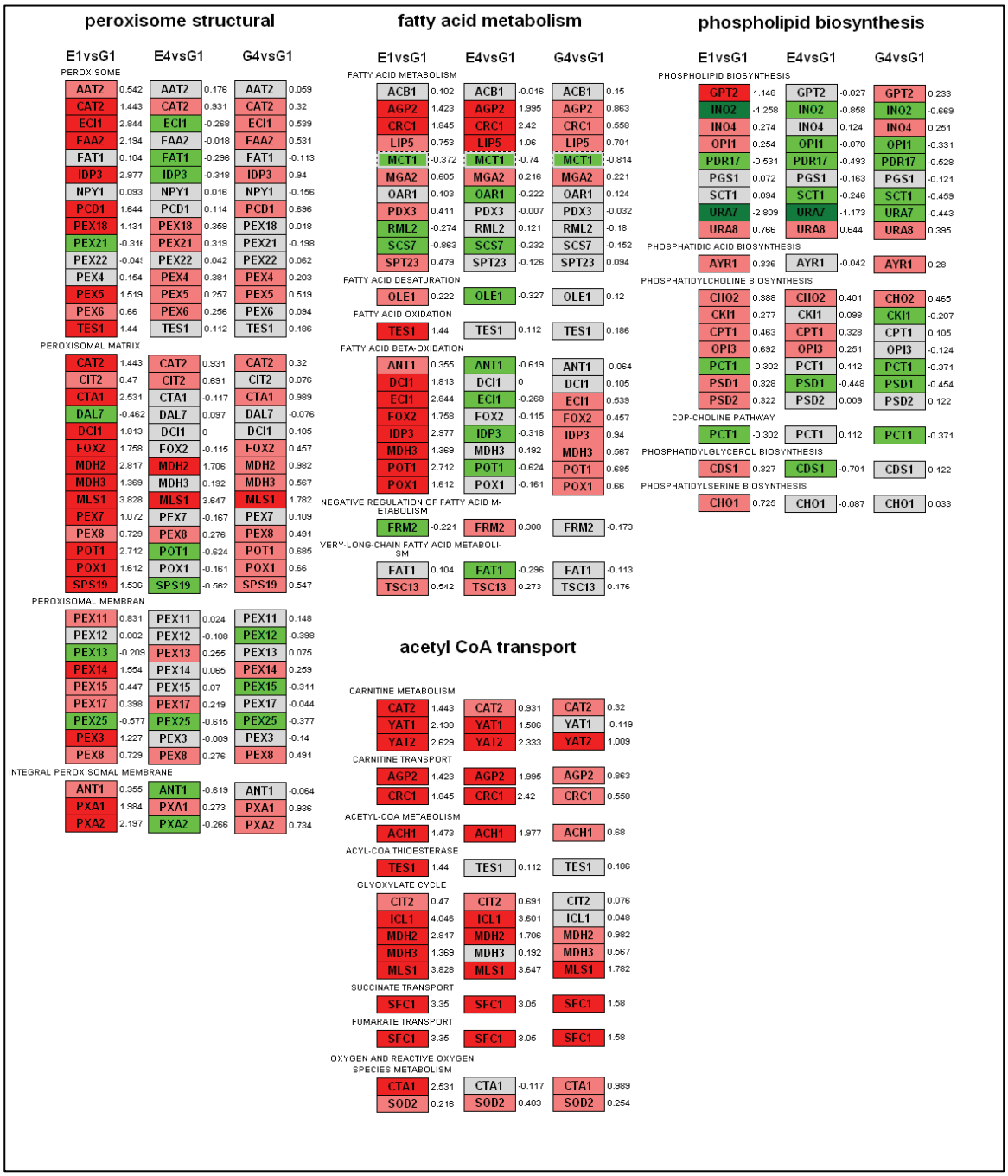

Figure 6.

Expression ratios of genes in the GO groups of “peroxisome”, “fatty acid metabolism”, “phospholipid biosynthesis”. Furthermore a selected group of genes that are mentioned in relation to acetyl CoA transport and carnitine metabolism[34,35,36,37]. All compared to standard glucose cultivation (G1). High up regulation in dark red, up regulation in light red. High down regulation in dark green, down regulation in light green. Grey: no criteria met. White: no data found. On ethanol all peroxisomal activity is up regulated, especially fatty acid beta oxidation. The resulting carnitine mediated acetyl-CoA transport is also strongly up regulated. Membrane phospholipid biosynthesis seems mildly up regulated under ethanol cultivation.

3. Redox balance and oxidative stress

As mentioned above the main NADPH and NADH generating steps during growth on ethanol are acetaldehyde dehydrogenation and the citric acid cycle. The redox balance in the cell is therefore substantially changed, which induces many metabolic changes (figure 7). This is illustrated by the strong up regulation of *NDE2*, a mitochondrial external NADH dehydrogenase that functions to supply any available cytosolic NADH to the mitochondrial respiratory chain. The change in redox balance might also be related to the strong up regulation of the NADH regenerating formate dehydrogenases *FDH1* and *FDH2*[40] (table II). Although no external formate is present, these up regulations on ethanol compared to glucose are very strong. This could be a result of the absence of glucose repression which has been described to be strong in methylothrophic yeasts and cannot be ruled out for *S. cerevisiae*[40].

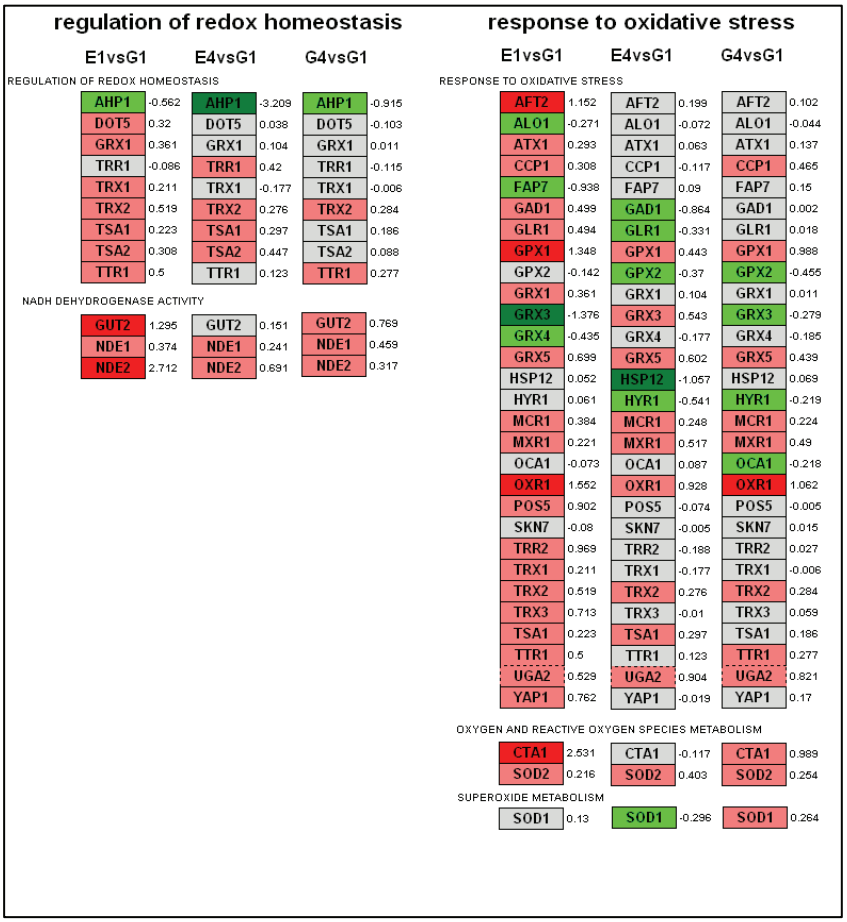

Figure 7.

Expression ratios of genes in the GO groups of regulation of "redox homeostasis" and "NADH dehydrogenase activity". And in GO groups of "response to oxidative stress" and "ROS metabolism". All compared to standard glucose cultivation (G1). High up regulation in dark red, up regulation in light red. High down regulation in dark green, down regulation in light green. Grey: no criteria met. White: no data found. On ethanol evidently there is up regulation of redox homeostasis and oxidative stress.

The changes in metabolism and redox balance, the peroxisomal activity and formation of reactive oxygen species (ROS) induce a strong oxidative stress on the cell when grown on ethanol (figure 7). Stress generally occurs when the cell detoxification is not capable of counter balancing these effects[39]. The response is apparent in the E1 samples, but under oxygen limitation in the ethanol decline phase (E4) or during the decreasing growth rate in glucose fermentation (G4) hardly present. Oxidative stress is mainly regulated by the transcription factor Yap1p, which is only induced in E1 samples. This does not imply that in the other phases oxidative stress is absent, as we have observed in an earlier study that production of a heterologous protein induces ROS formation and even oxidation of important proteins present in the ER[32]. Redox balance, oxidative stress and ROS are involved in protein folding processes in the ER[41] and the unfolded protein response. This is due to the strong dependency of protein folding in the ER of the GSH:GSSG ratio, as well the activity of disulphide isomerises and the use of peroxide and superoxide as electron acceptors[41,42].

4. Protein metabolism

Notable differences in the expression of protein catabolism genes are observed between E1 and G1 during production of a heterologous protein (table II).

The large up regulation of genes encoding proteins of the autophagy and vacuolar targeting processes on ethanol and the absence of this up regulation in glucose grown cells indicates that this is mainly an ethanol effect. Furthermore, this up regulation (figure 8) indicates a high turnover of proteins and thus generation of amino acids when *S. cerevisiae* is grown on ethanol. This is further supported by the up regulation of many steps in the central nitrogen metabolism and the deamination of certain amino acids, as described below.

Also, the ubiquitin cycle and the 26S proteasome are strongly up regulated. Most likely the turn over of a number of nuclear and cytoplasmic proteins is increased by degradation via the proteasome (also strongly up regulated), in spite of the considerable energy costs of these processes. The latter protein catabolic processes are directly linked to unfolded protein response UPR[4,6] and Endoplasmatic Reticulum Associated Degradation (ERAD)[33,43]. The associated GO annotations (figure 9) however, are not significantly up regulated.

This could be caused by the transient response in long term ethanol cultivation. As cells are subjected to the stress for a longer period the effecting response could become weaker[10].

HAC1, encoding the main transcriptional factor for unfolded protein response is stronger expressed at a higher level in glucose than on ethanol. The UPR is also induced as a result of heterologous protein production and therefore already up regulated under normal glucose growth. This has been described for the production in a very similar production system by Sagt *et al.*[32] for another protein, the folding of which was impaired by site directed mutagenesis.

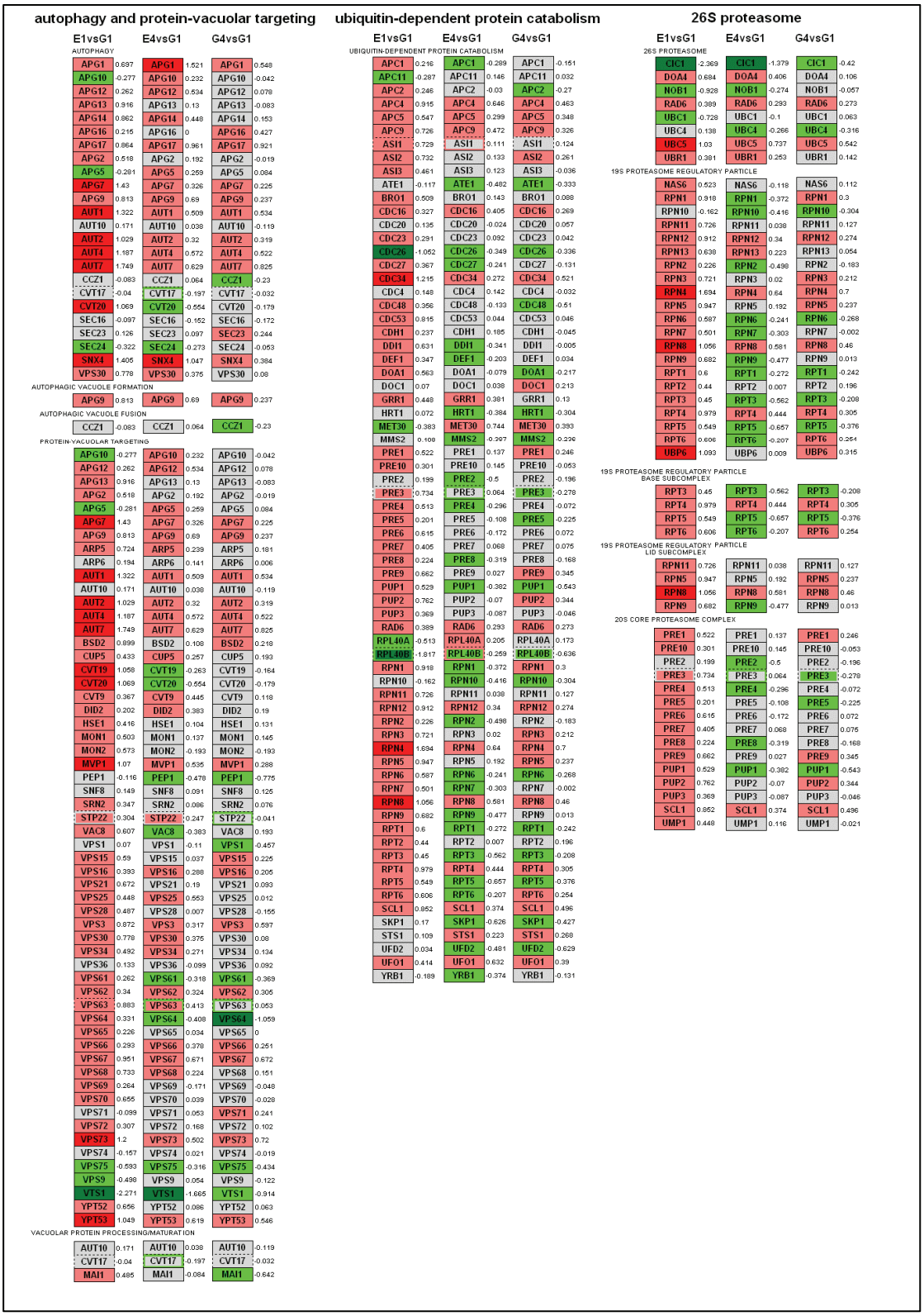

Figure 8.

Expression ratios of genes in the GO groups of “autophagy” and “protein vacuolar targeting”, “ubiquitin dependent protein catabolism” and “26S proteasome”. All compared to standard glucose cultivation (G1). High up regulation in dark red, up regulation in light red. High down regulation in dark green, down regulation in light green. Grey: no criteria met. White: no data found. On ethanol all catabolic processes are strongly up regulated.

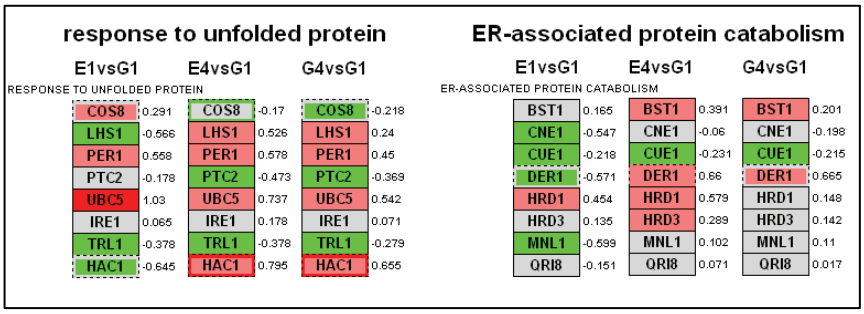

Figure 9.

Expression ratios of genes in the GO groups of "Response to unfolded protein" and "ER associated protein catabolism". All compared to standard glucose cultivation (G1). High up regulation in dark red, up regulation in light red. High down regulation in dark green, down regulation in light green. Grey: no criteria met. White: no data found. On ethanol the unfolded protein response is neutral compared to glucose, probably of the inherent induction by heterologous protein production. ER-associated protein catabolism appears even to be down regulated on ethanol (E1) but mildly up regulated under ethanol stress.

5. Amino acid, co-factors and nucleotides

In figure 2 the down regulation of pyruvate decarboxylases (*PDC1*, *PDC5*, *PDC6*) on ethanol cultivation is visible. *ARO10* however is up regulated and in the ethanol decline phase very strongly up regulated. This decarboxylase and *THI3* are related to amino acid catabolism[28] and fusel alcohol production[44] which also consists of the aromatic amino transferase Aro9p and the branched chain amino transferases Bat1p and Bat2p[45]. In figure 11b the response levels of these genes are also shown. The up regulation is only visible in E1 and E4 and therefore seems to be an ethanol effect. As described by Eden *et al.*[46] *BAT1* is repressed and *BAT2* is induced during stationary phase or at the low growth rate. *ARO9* and *ARO10* are regulated by the same transcription factor *ARO80* which is also up regulated upon ethanol cultivation and contains several motifs that are analogous to *GAL4* transcription factor[47]. Moreover, the amino acid catabolic activity of Aro9p is sensitive to nitrogen catabolite repression, which is strong under these conditions with ammonia as nitrogen source. Therefore the direct reason for up regulation of this group of genes can be many fold but seems to be more related with up regulation of protein catabolic processes and nitrogen metabolism than with carbon metabolism.

The main nitrogen source in the fermentations in these studies is ammonia in relatively high concentrations (between 15-75 mM) (figure 10).

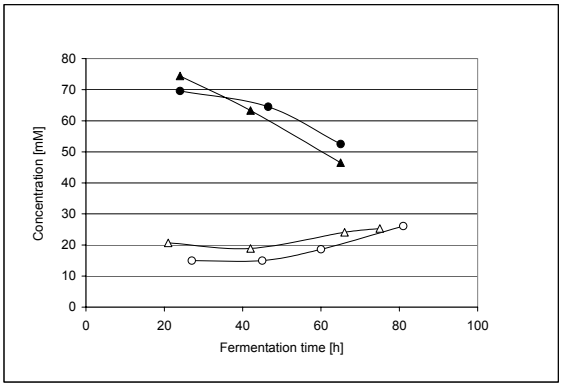

Figure 10.

Ammonia concentration in fed-batch fermentations on glucose (●) and ethanol (○) and phosphate concentrations on glucose (▲) and ethanol (△) with *S. cerevisiae* producing VHH-BC15.

Furthermore, amino acids are available beyond limiting amounts, as 50% reduction of the yeast extract did not alter biomass and heterologous product yield (data not shown). Due to the high ammonia concentration the intracellular amount of ammonium and thus glutamate is probably high which induces nitrogen catabolite repression on amino acid metabolism and transport (table III)[48,49].

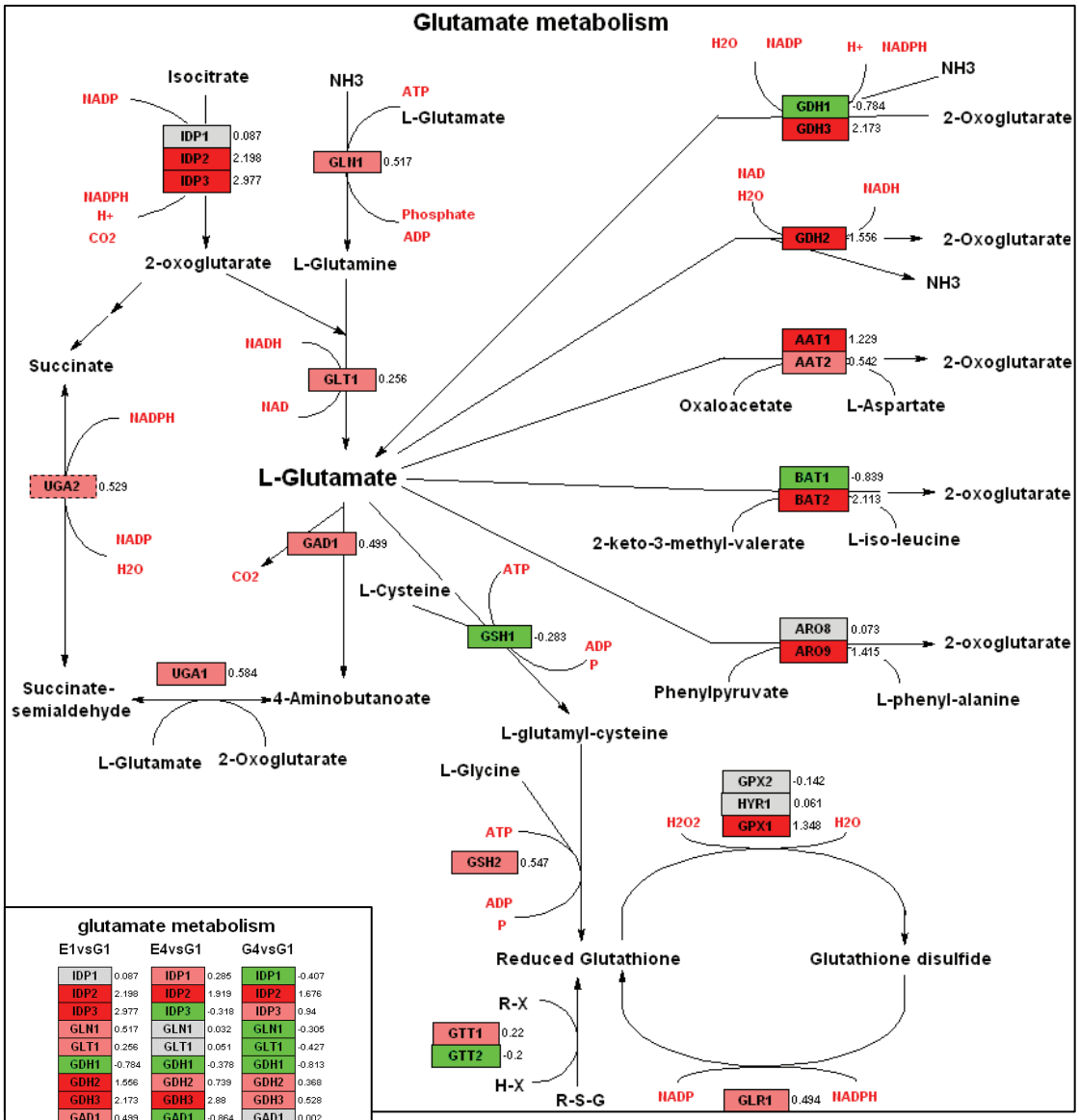

Figure 11.

A: E1vsG1 expression ratios of genes involved in central nitrogen and glutamate metabolism. Comparison of ethanol (E1) and glucose cultivation (G1) during the exponential growth phase in fed-batch fermentations with *S. cerevisiae* producing VHH-BC15. The pathway model is based on data from SGD[18] and KEGG[60].

B: All expression ratios of the selected genes mentioned in the central nitrogen and glutamate metabolism. All compared to standard glucose cultivation (G1).

On ethanol the glutamate consumption towards 2-oxoglutarate seems to be more strongly up regulated. This effect is most strongly in ethanol cultivation (E1).

Apparently under ethanol instead of glucose growth, the nitrogen metabolism changes severely. In figure 11a the more detailed picture of the Central Nitrogen Metabolism is given. It has been described that ethanol cultivation decreases glutamate dehydrogenase Gdh1p activity and increases Gdh3p activity[50], which is confirmed in the figure. Gdh3p has a low affinity for 2-oxoglutarate[51] and thus the reaction towards glutamate is less favourable. Moreover *GDH2* becomes up regulated upon glutamate accumulation[48,50], converting it into 2-oxoglutarate, a key metabolite in the TCA. Also aminotransferases Aat1p, Aat2p, Bat1p, Bat2p and Aro9p are able to convert glutamate to 2-oxoglutarate thereby donating amino groups for the amino acid biosynthesis, which are all up regulated. The ethanol metabolism of *S. cerevisiae* in a medium containing ammonia in a non limiting concentration, apparently uses this nitrogen source for replenishing the TCA via glutamate conversion.

6. Vitamins

Ethanol cultivation (table II) also shows a clear up regulation of vitamin metabolism such as biotin. Biotin is a cofactor in pyruvate carboxylase (Pyc1p) and thought to play a role in the regulation of glucokinase (Glk1p) and phosphoenol pyruvate carboxykinase (Pck1p) and is thus important for gluconeogenesis and ethanol metabolism[52]. The rate-limiting enzyme in biotin synthesis is the mitochondrial Bio2p that contains a 4Fe-4S cluster[53] which links biotin to respiration and metal dependency. Moreover the mitochondrial respiratory chain requires additional iron due to the oxidative stress[54]. The strong up regulation of biotin metabolism however is also witnessed in the decline phases of ethanol and glucose cultivation. Apparently this is related to a decreased growth rate.

7. Stresses and low growth rate

On ethanol, the stress response is overall clearly up regulated as expected (figure 12)[4,6]. In a comparison with the STRE-regulated genes described by Moskvina *et al*.[55] 70% of these stress responsive genes were also up regulated in our data set above an expression ratio of 0.2. This implies that even under the long-term transient conditions of our experiments still a general stress response is evident.

Highly up regulated are genes related to trehalose (*ATH1*, *TSL1*), starvation (*XBP1*), catabolism (*UBC5*) and heat shock proteins *SSA3* and *HSP82*, which are directly linked to protein folding and proteasomal induction. Also down regulation is visible: the osmotic responsive genes *DOG2*, *GRE1*, *GRE2* and *LTV1* and the cytosolic catalase *CTT1*.

Poor availability of nutrients slows down the growth rate and induces finally the stationary phase. These processes are mainly regulated by the TOR pathway[56,57]. Most important is that this pathway decreases protein synthesis by a significant decrease of the transcription of the ribosomal proteins. This is most clearly witnessed for E1 vs G1 and also for E4 vs G1 but hardly for G4 vs G1 (table III). As the growth rate for G4 and E4 is the same (μ=0.03) and for E1 even larger (μ=0.06) this down regulation cannot be solely assigned to the growth rate and it has to be concluded from our data that the synthesis of ribosomes is clearly down regulated

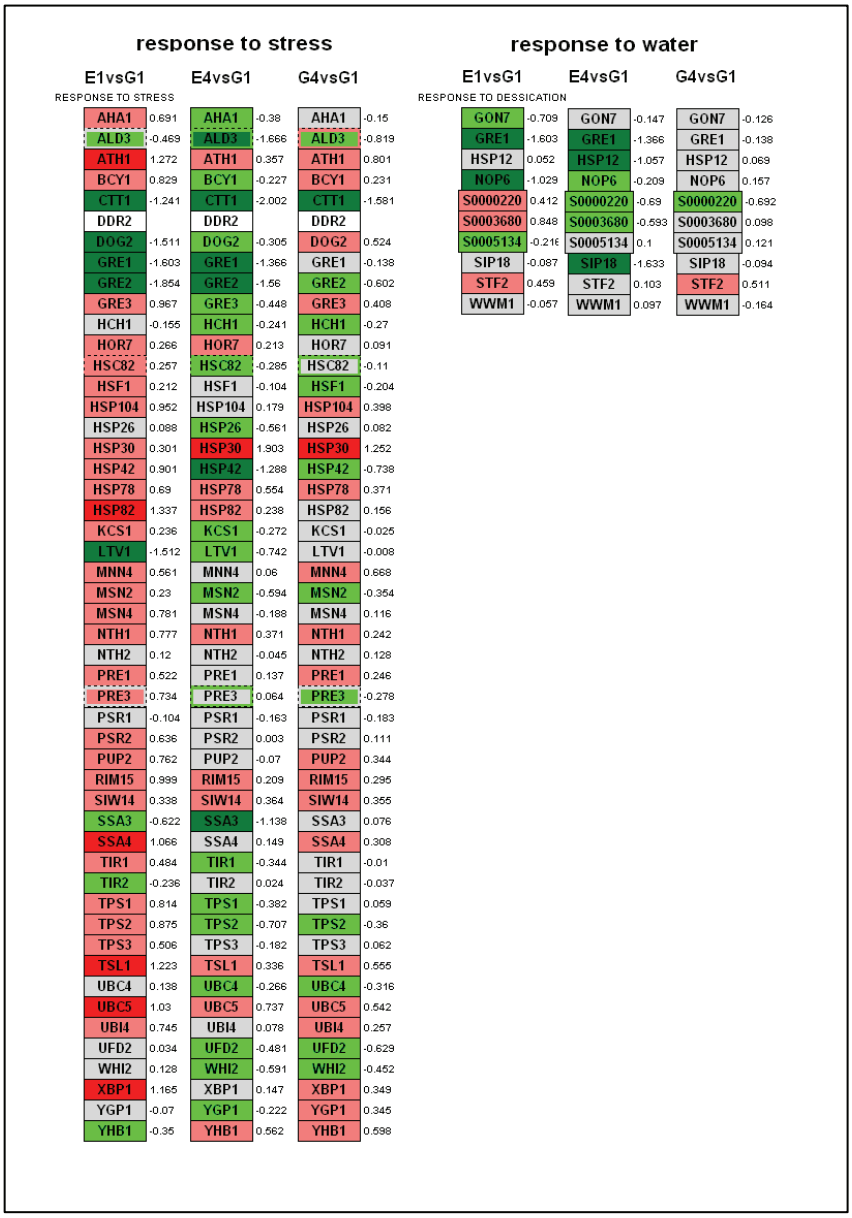

Figure 12.

Expression ratios of genes in the GO groups of “Response to stress” and “response to water”. All compared to standard glucose cultivation (G1). High up regulation in dark red, up regulation in light red. High down regulation in dark green, down regulation in light green. Grey: no criteria met. White: no data found. On ethanol the stress responses are more evident compared to glucose. The response to water deprivation is evidently down regulated in the final ethanol phase (E4) compared to glucose.

on ethanol growth. The down regulation of translational capacity has also been linked to ER-stress[58] indicating to increased protein folding and protein catabolic activity. As this transcriptional and translational down regulation on ethanol has no consequence for the heterologous protein secretion it has to be concluded that on glucose there is a surplus on ribosomal capacity.

Comparison E4 and G1

1. Carbon metabolism

The changes observed in expression of genes encoding proteins of the carbon metabolism (E1 vs G1) are also present for E4 vs G1, indicating that there is no significant change in carbon metabolism due to the reduction in growth rate and the low oxygen tension of the medium (table II).

Remarkable are the genes encoding for galactose metabolism that are more strongly expressed in E4 than E1 (figure 4). This could mean that in ethanol limited cultivation (E1) still a limited glucose repression is present, albeit much less than in G1. But in glucose limited cultivations the residual glucose in the medium is neglectable and in ethanol cultivation completely absent. However, glucose repression could be also caused internally through the catabolism of storage carbohydrates. Figure 5 shows that the glycogen and trehalose metabolism is stronger up regulated in de E1 phase than in E4. Because of this and the decreased growth rate in E4, the average internal glucose concentration would be lower in E4. This further glucose de-repression in the ethanol decline phase is also supported by the strong down regulation of the glucose metabolism and transport in E4 (table III). It also implies that in the E4 phase the transcription of the gene encoding VHH-BC15 from the *GAL7* promoter is higher, indicating that even at very low glucose concentrations as are present in G1, there is some glucose repression.

2. Peroxisome

The GO annotations related to peroxisomes and fatty acid oxidation are not as strongly up regulated anymore or even slightly down regulated in the decline phase on ethanol (figure 6). Apparently the decreased growth rate doesn't result in further proliferation of the peroxisomes. The peroxisomal export of acetyl-CoA however seems still highly active, as both carnitine metabolism and glyoxylate cycle are highly up regulated.

3. Redox balance and oxidative stress

Whereas in the E1 phase of growth the genes encoding proteins to neutralise or at least minimise the detrimental effects of reactive oxygen species are strongly up regulated (figure 7) this up regulation is hardly noticeable in E4. This is in line with the expression of *YAP1*. Yap1p is the main transcription factor regulating response to reactive oxygen species and is not up regulated in E4. The same holds for the expression of important enzymes in the neutralisation of reactive oxygen species like Trx2p, Cta1p and Sod1p. The reason for this neutral response in E4 could be analogous to the decreased peroxisomal activity, due to lower metabolic activity and the adaptation to the ethanol cultivation.

4. Protein metabolism

Figure 8 illustrate very clearly the large difference in expression of genes encoding for proteins involved in protein catabolism. The strong up regulation in the first phase of ethanol

cultivation (E1) is not apparent anymore in the final phase. Although autophagy and vacuolar targeting are still moderately up regulated, the ubiquitination and proteasome are neutral or even down regulated. Apparently, similar to the peroxisomal response, the decreased growth rate reduces the general transcriptional activity. Furthermore the long-term ethanol cultivation has made the cells adapted well enough to cope with protein catabolism.

Remarkably the unfolded protein response and the ER associated degradation seem stronger up regulated in the E4 phase. The presence of incorrectly folded proteins under a decreasing growth rate is energetically very unfavourable which could induce the up regulation of *HAC1*. Whether the observed down regulation of the protein catabolism and the induced UPR has a direct effect on the specific higher production of Camelid antibodies growing under the E4 vs the E1 conditions is presently under investigation.

5. Amino acid, co-factors and nucleotides

A logical consequence of the reduction of transcription of genes encoding for 26S proteasome, autophagy and ubiquitin-dependent protein catabolism in the E4 phase, should be a reduction in certain parts of the Central Nitrogen Metabolism. Indeed, as illustrated in Fig 11b, with the notable exception of *IPD2*, *BAT2*, *ARO9* and *ARO10* most of the other genes are less transcribed under E4 than E1 conditions. However in table II, a strong up regulation is noticeable for the GO annotations of general transport and more specific transport of amino acids. Furthermore, several parts of the amino acid metabolism are also strongly up regulated. This could be due to a lower nitrogen catabolite repression and a shortage of amino acids for protein synthesis by a reduced protein catabolic activity. Then apparently transport is strongly induced to counter this effect and the extracellular medium is the source for additional nutrients.

6. Vitamins

The same effect as for amino acids is seen with the vitamins (table II). A shortage of building blocks and co factors induces transport and metabolism of not only biotin and carnitine, but also thiamine, pyridoxines and sulphur metabolism are strongly up regulated in the final phase of ethanol cultivation.

7. Stress and low growth rate

The reduction in growth rate by oxygen limitation and ethanol inhibition in the E4 phase also shows a strong down regulation of transcriptional activities induced by TOR (table III). Again, this effect is less strong in the E4 phase than in the earlier ethanol cultivation phase due to the lower metabolic rate, compared to normal growth on glucose (G1). This is also evident in the up regulation of mating related functions that are induced upon a reduction in growth rate (table II).

A remarkable result is the strong down regulation of genes related to water deprivation and desiccation (*GRE1*, *HSP12* and *SIP18*) in the decline phase of ethanol cultivation (figure 12).

These genes code for hydrophilic proteins that are induced under hyperosmotic conditions or low water concentrations and are related to the HOG-pathway[59]. In figure 12 the up regulation is clearly seen for the final phase of ethanol cultivation (E4vsG1) but also in the E1 phase there is some general up regulation of these genes visible. This implies that under normal glucose cultivation with relatively higher biomass and high medium concentrations the osmotic stress is stronger than under ethanol cultivation especially under 1%v/v ethanol accumulation. Figure 10 illustrates this as both ammonia and phosphate concentrations are three times higher in the early glucose cultivation.
In general however the stress response of the late ethanol cultivation is lower than in earlier ethanol cultivation and in some aspects even strongly down regulated (figure 12). This is probably due to the decrease in growth and the transient change in response. As discussed before the genomic expression adapts to the environmental change in time by lowering the response[10].

Comparison G4 and G1

1. Carbon metabolism

The main difference between G4 and G1 is the growth rate. Therefore no significant changes in the carbon metabolism are expected as indeed proved to be the case (table II and III). Glucose transporters are however up regulated. Also the expression of the galactose related genes are up regulated in the decline phase on glucose (figure 4). This was also seen for the ethanol cultivations and seems to relate more with decreasing growth rate than with glucose derepression. It could be that besides the glucose transport also galactose is now induced to compensate for the loss in carbon flux in the limiting end phase, although this doesn't effect in any additional carbon due to the *GAL1* deletion. This is also evident in the generally up regulated energy reserve metabolism (figure 5).

2. Peroxisome

In tables II and III no high up regulation of peroxisomal activity is seen. However, the GO annotation in figure 6 shows some up regulation of the peroxisomes and the beta oxidation. There is however no lipid biogenesis and it therefore seems more related to additional carbon and energy generation against the decreasing metabolic rate, by lipid turn over.

3. Redox balance and oxidative stress

The cultivation on glucose under lower growth rate does not seem to induce elevated levels of oxidative stress. In this aspect the response is very much like the E4 response. There is however some up regulation NADH dehydrogenase activity and ROS, probably induced by peroxisomal induction (figure 7).

4. Protein metabolism

The response ratios for protein catabolic processes are more or less neutral for G4 compared to G1. Only some autophagy seems up regulated as response to decreasing growth rate. The strong up regulation of protein catabolism seems very much an ethanol effect and not because of the energetic and metabolic burden of heterologous protein production.

5. Amino acid, co-factors and nucleotides

As can been expected at very low growth rates, the incorporation of ammonia into glutamate is significantly reduced as transcription of *GLN1*, *GLT1* and *GDH1* are reduced, even stronger than the reduction of the transcription of these genes at the same growth rate grown on ethanol. In general the whole amino acid metabolism is strongly reduced in activity (table III). Apparently the need for amino acids is low at these reduced growth rates and a decreased heterologous protein production.

6. Vitamins

As described earlier the up regulation of biotin related genes in the decline phase of glucose indicates to a medium shortage more than to a growth rate or ethanol effect (table II).

7. Stress and low growth rate

Also the stress response is more neutral than in ethanol cultivations. *HSP30* is strongly up regulated (figure 12) in the E4 and G4 phases but this plasma membrane heat shock protein is also induced upon stationary phase stress. The reduction in growth rate however seems to be a direct influence for a mating-pheromone up regulation as this is both witnessed for E4 and G4 versus G1.

Discussion

DNA arrays are a powerful tool for fundamental as well applied studies. We observed that *Saccharomyces cerevisiae* cultivated in fed-batch fermentation at low growth rates produce much higher amounts of heterologous proteins per time and biomass on ethanol than under similar conditions on glucose. This was not only observed when variants of the Camelid antibody fragment VHH-BC15 was produced, but also for completely different VHH's or even completely different proteins like the anti freeze peptide[1]. To investigate this unexpected result and the cell state causing it, we performed DNA array analysis. As the literature provide a large amount of DNA array data of *S. cerevisiae* cultivated under various conditions on glucose[4,6-11] we used that as reference for our study. This study concentrated on pair wise analysis of array data obtained early and at the end of the fed-batch process using either glucose or ethanol as main carbon source.

Whereas many of the data we presented in this study are in agreement with literature data and therefore not discussed here in detail, a number of new observations were done as well.

The carbon metabolism changed mainly as expected. However, galactose genes were strongly up regulated under ethanol cultivation and low growth rate, in spite of a constant expression of the key regulator of the galactose metabolism, Gal4p[22]. This pointed in the direction that under those conditions glucose repression was really eliminated.

Another notable observation is that the peroxisome was well proliferated and genes encoding for fatty acid degradation were strongly up regulated. Peroxisomal activity generates reactive oxygen species and aldehyde intermediates that induce respectively oxidative stress and unfolded protein response. Furthermore the fatty acid oxidation generates additional carbon and energy for TCA activity, which is essential for metabolic activity. It has been described that the UPR initiate the synthesis of components for the (ER) membrane[32,33]. Because of the adverse effects of ethanol on membrane structures it may be that the turnover of phospholipids is higher under ethanol conditions, which also contribute to an increase in oxidation of fatty acid.

The redox potential in *S. cerevisiae* growing on ethanol is very different from growing on glucose. The first steps of the ethanol metabolism as well the enhanced activity of the citric acid cycle create a more reducing environment. This change in redox balance has a strong influence on respiration capacity and the cell state. Furthermore it has been described in detail that the right redox potential is essential for correct protein folding in the ER[41]. It may be that when grown on glucose the ratio NADH:NAD in the ER is too low, whereas when reducing agents like DTT are added to the medium this ratio is too high. This would impair protein folding, whereas for ethanol this ratio is maybe optimal for protein folding. The increased pool of cytosolic NADH could also increase the rate of regeneration of GSSG to GSH, which is necessary for optimal protein disulphide isomerase activity. That the redox balance is important is clearly demonstrated in an earlier study of us, where variants of VHH's containing a free cysteine are poorly produced on glucose, but quite well on ethanol[1].

In the nitrogen metabolism it is evident that on ethanol cultivation the main nitrogen source ammonia, is directed towards the TCA for replenishment of carbon and energy metabolism. This is coupled to a strong nitrogen catabolite repression on amino acid metabolism. However in the final phase of ethanol cultivation under a decreasing growth rate the amino acid transport and metabolism is induced, to overcome amino acid shortage. This could be one of the reasons for the further improved heterologous protein production in this final phase of ethanol cultivation as described in earlier work.

However the most remarkable result is that processes that break down proteins like autophagy and proteasome mediated processes are very strongly up regulated when ethanol was used as main carbon source while the production of the heterologous protein was higher. This up regulation is even more pronounced as for the production of a heterologous cutinase mutant that was badly secreted[32]. For cutinase we performed pulse chase analysis and the

results clearly pointed in the direction of an extensive breakdown of this protein via the ERAD/proteasome pathway[32]. DNA arrays do not provide sufficient information to explain this and therefore in following study pulse chase experiments will be performed to see whether growth on ethanol results in a relative lower rate of improper folding and subsequent degradation of the heterologous protein.

Although our cultivation conditions are different from the continuous cultivation of *S. cerevisiae* on ethanol performed by Daran-Lapujade[11] *et al*. we made a qualitative comparison of their and our study in which the main difference is the production of a heterologous gene in our strain. Compared to the very high up regulation of the GO groups proteasome, ubiquitin system, autophagy and vacuolar catabolism in our data set, the data of Daran-Lapujade *et al*. shows a general up regulation of autophagy and vacuolar catabolism, stronger up regulation of the ubiquitin system but a neutral response in the proteasome group. So it can be concluded that ethanol induces ubiquitin mediated protein catabolism and to a smaller extend autophagy and vacuolar protein breakdown, but that protein catabolism is much stronger due to the production of heterologous proteins.

Finally this study confirms earlier observations that low growth rates result in a redirection of the scarce energy and nutrients from producing more ribosomes and other main cellular proteins to protein secretion.

Based on our data it can generally be said that the two main up regulated effects in the cellular response to ethanol are catabolism and transport to relieve the cell of harmful intermediates and to replenish the central carbon and energy metabolism. This response is strongest in the ethanol limited exponential phase of the fed-batch fermentation and becomes more transient but still effective in the decline phase. To further clarify this mechanism this DNA microarray and additional data will be studied by focusing on the protein production and secretion cascade in future work.

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CHAPTER 5

An integrated analysis of the increased secretion efficiency of heterologous proteins by *S. cerevisiae* in ethanol cultivations

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Abstract

The analysis of mRNA profiles of heterologous protein producing *S. cerevisiae* when grown at ethanol and glucose in a fed-batch process is complicated as various parameters are more or less simultaneously changed. However this is inherent to fed-batch processes and as they are the commonly used fermentation processes in industry, there is a clear need to understand such a complex process. GO analysis of all array data showed that several different metabolic and physiological groups were strongly affected by the shift of glucose to ethanol. Changes in expression profiles of gene encoding enzymes of glycolysis, TCA cycle and uptake and conversion of ethanol into acetyl-CoA were expected, however the large increase in protein catabolism, proteins involved in the cellular redox system and the lack of change in UPR/ERAD were less expected.

Certain results of the DNA arrays have been verified with independent techniques, like Northern analysis for the determination of the efficiency of transcription process. Also with pulse chase experiments to determine turn over of proteins, as a high protein catabolism was one of the main characteristics of *S. cerevisiae* grown in a fed-batch process and producing a heterologous gene product. Finally, electron microscopy was performed to determine aberrant intracellular structures and the intracellular localization of the heterologous protein and the ER resident BiP.

The information of these studies and the DNA arrays have been used to analyse the relative in- or decrease in each step in the cellular process involved in heterologous protein production. Integration of these steps creates the following picture of the difference of the two carbon sources:

a. transcription of mRNA encoding VHH-BC15 increase when grown on ethanol;
b. translation of this mRNA is reduced;
c. correct folding of VHH-BC15 in ER is significantly improved;
d. the trans Golgi transport is increased
e. VHH-BC15 seems to exocytosed when *S. cerevisiae* grows at low growth rate on ethanol. However the exocytosis is not complete as VHH-BC15 is clearly present in the enlarged cell wall.

Introduction

For the production of heterologous proteins as well for the production of biomass or metabolites, fed-batch processes are the preferred process in industry. Recently we have found that the yield of heterologous proteins produced by *Saccharomyces cerevisiae* in fed-batch fermentations with ethanol as main carbon source was significantly higher than the production of the same heterologous protein in fed-batch processes with glucose as main carbon source[1].

There are several major differences of cellular processes between glucose and ethanol based fed-batch cultivation, notably (i) a highly altered carbon metabolism and reserve metabolism, yielding less biomass and a lower growth rate[2-4]; (ii) a change in redox balance[2,5]; (iii) physiological effects like membrane permeabilisation and reduced viability[6] and (iv) the induction of several stress responses[6,7].

During the transition of the exponential phase to the final phase of a fed-batch process, the growth rate drops considerably but production of heterologous proteins remained higher in the final phase of the ethanol process while on glucose the specific production decreased. For a better understanding of the cell status under these conditions genome wide DNA arrays were performed[8].

In addition to stress, metabolics and redox balance, catabolic processes had a large impact on the cell status. To understand how this cell status on ethanol cultivation can increase the heterologous protein production a further investigation of the microarray data was performed. The whole cellular production process of the heterologous protein was therefore analysed ranging from transcription to protein folding and secretion.

In this study we found that several stress effects and the redox state co-induce a much more efficient secretion process through (re)folding capacity increase, resulting in a higher heterologous protein flux. A model in which the various observations are integrated will be discussed.

Materials and methods

Strain

Saccharomyces cerevisiae strain VWk18 *gal1, leu2*, carried a multi copy integration vector integrated at the rDNA locus[9], containing the heterologous protein coding sequence, linked to a signal sequence and under control of the *GAL7* promoter and *leu2d* selectable marker to maintain a high copy number. The strains used in this work are CEN.PK102-3Agal1 which produces the llama antibody fragment VHH-BC15 linked to a Hmss1 signal sequence[1] and the *pmt1* strain derived from CEN.PK338 which produces the antifreeze protein (AFP) linked to an invertase (*SUC2*) signal sequence.

Fed-batch fermentations

The rich medium used for preparation of the inoculum and in the fed-batch fermentations is described in van de Laar *et al.*[1]. The fermentations were performed in standard fermenters with a 10 L working volume according to the same protocol. The batch phase (30°C, 2 L/min air, DO_2 minimum 30%, pH 5.0) was started by inoculation with 500 ml full-grown YPD culture (1% yeast extract, 2% peptone, 2% glucose). The feed was automatically started when the

ethanol concentration decreased below 300 ppm. In the feed phase (21°C, 6 L/min air) the exponential feed rate was applied to maintain a growth rate of 0.06 h^{-1}. In the glucose fermentation, the feed rate was set to linear when the DO_2 level decreased below 15%. In the ethanol fermentation the DO_2 decreased to 0% and subsequently accumulating ethanol increased to a maximum of 1500 ppm in the off-gas. Then, to maintain this accumulation between 800 and 1500 ppm a pulsed feed profile was applied.

Sampling, RNA isolation and microarray

Samples for microarray analysis were taken at two different time points from a glucose (G1 and G4) and an ethanol (E1 and E4) fed-batch fermentation. The samples were taken directly from the fermenter and immediately frozen in liquid nitrogen. The time points of each sample correspond to the exponential phase after approximately 26 hours (G1 and E1) and decline phase after approximately 66 hours (G4 and E4). An overview of the samples and the characteristics is given in table I.

For RNA isolation, samples were thawed on ice and approximately 100 mg cells were resuspended in 12 ml AE-buffer (50 mM NaAc pH 5.2, 10 mM EDTA). RNA extraction was performed by the method of Llinas[10]. The purity and integrity of the samples were verified by an RNA labchip 2100 bioanalyzer from Agilent technologies. Before hybridisation the 4 regular samples were treated and their quality verified according to Affymetrix protocols[11]. DNA microarrays were performed by hybridisation of the samples to the Affymetrix GeneChip Yeast Genome S98 Array according to Affymetrix protocols. The arrays were scanned by a GeneArray Scanner system and data were extracted with microarray Suite 5.0.

Data processing

Raw data were analysed in dChip as described by Zakrzewska *et al.*[12] for assessing standard errors and normalising the data. The 4 data sets were together analysed against the G1 sample. The obtained expression levels were converted into an MS excel format and expression ratios based on ^{2}Log values were calculated. The following response criteria for the expression ratios were used: high down regulation (ratio ≤ -1), down regulation (-1 < ratio ≤ - 0.2), no response (-0.2 < ratio < 0.2), up regulation (0.2 ≤ ratio < 1), high up regulation (ratio ≥ 1).

Function classes for the identified genes were determined based on Gene Ontology annotations[13] in the *Saccharomyces* genome database (SGD)[14] and GenMAPP[15], a gene map annotator and profiler program.

Northern blot analysis of VHH-BC15 and ACT1 mRNA

For northern blot analysis, 10 µg of total RNA per sample was resolved on a formaldehyde agarose gel and transferred to Hybond-N+ membrane (Amersham Biosciences) by capillary transfer using 20 × SSC (3 M sodium chloride, 0.3 M sodium citrate) according to the

manufacturer's instructions. After blotting overnight, ribosomal bands were visualised by methylene blue colouring and the result was scanned. For the detection of the mRNA transcripts, the membrane was incubated at 65°C for 1 hour with 6 × SSC supplemented with 5 × Denhardts solution (0.5% (w/v) Ficoll PM400, 0.5% (w/v) polyvinylpyrolidone and 0.5% (w/v) BSA), 0.5% SDS and fragmented herring sperm ssDNA (0.1 mg/ml). Subsequently, hybridisation was performed with ^{32}P-labeled probes directed either to VHH-BC15 mRNA or to *ACT1* mRNA at 42^{o}C overnight in a solution of 50% (v/v) formamide, 10% (w/v) dextran sulfate, 6 × SSC, 2 × Denhardts solution and 0.1% (v/v) SDS. The next day, the membrane was washed twice at 55°C for 20 min with 2 × SSC containing 0.5% SDS, followed by washing the blot twice for 20 min at 55°C with 0.2 × SSC and 0.5% SDS. Subsequently, the membrane was exposed to a phosphor screen (Molecular Dynamics) overnight. Between the hybridisations the membrane was stripped with boiling 0.1% SDS followed by a brief wash in 2 × SSC at room temperature. The 350 bp *Pst*I-*Bst*EII *VHH-BC15* gene fragment and the 1.5 kb *Bam*HI-*Hin*dIII *ACT1* gene fragment were used as templates for the generation of random labelled probes with the Prime-a-Gene kit (Promega). For quantification of the mRNA transcripts, the phosphor screen was scanned using a personal FX Typhoon scanner (Bio-Rad), and the relative levels of VHH-BC15 and *ACT1* mRNA were determined by comparing the band intensities to that of 18S RNA using the Quantity software (version 5.1; Bio-Rad).

Internal product analysis by western blotting

For the determination of the internal product several samples were taken from the fermenter. Cells were spun down at 13,000 rpm for 4 minutes and washed 3 times with 1x PBS pH 5.0.
Of this cell pellet, 50 mg was resuspended in 0.5 ml 1x PBS pH 5.0 and transferred to a glass tube and the same volume of glass beads (425-600 microns acid washed, Sigma) was added and vortexed 4 times 30 seconds for efficient cracking of the cells while keeping on ice in between.
0.5 ml 1x PBS pH5.0 was added and the supernatant was centrifuged at 13,000 rpm for 4 minutes. The soluble fraction (supernatant) was then run on an SDS page gel (10-20% Tricine gels, Novex) and subsequently transferred to a PVDF membrane (immobilon P transfer membrane, Millipore) in a Novex blotting module for 1 hour at 325 mA in 1x blotting buffer (20% methanol, 0.19 M Glycine, 25 mM Tris, 0.02% SDS). The membrane was blocked by 4% Marvel milkpowder in PBS at room temperature and subsequently incubated in 2% Marvel in PBST (0.05% Tween20 in PBS) containing 2000x diluted rabbit anti-llama IgG serum K208 and after washing in PBST incubated in 2% Marvel in PBST containing 2000x diluted anti-rabbit IgG conjugated to AP (Promega). The protein was then detected by a BCIP-NBT colourmetric reaction according to the manufacturers manual (AP conjugate substrate kit, Bio-Rad). The total protein of the solubilised fraction was determined by Bradford analysis.

Pulse chase experiments

Yeast cells grown in fed-batch cultures were washed twice in YNB-2%galactose (YNB-Gal) and resuspended in 4 ml YNB-Gal to a final OD_{600} of 10. After incubation for 1 h at 30°C (phase I samples) or at 22°C (phase II samples), cells were labelled for 10 min with 580 µCi of [^{35}S]methionine-cysteine (Redivue Pro-mix; Amersham Biosciences). Chase of radiolabelled proteins was performed by washing the cells in 1 ml of YNB w/o amino acids-Gal, followed by resuspension of the cells in 4 ml YNB w/o amino acids-Gal, and adding 800 µl of a nonradioactive mixture of methionine (100 mM) and cysteine (50 mM). At the indicated times, 800 µl samples were centrifuged and cells were lysed by addition of 480 µl of 1.85 M NaOH–7.5% β-mercaptoethanol, followed by incubation on ice for 10 min. After precipitation of the proteins from the supernatant or of the cell fraction with 10% trichloroacetic acid (TCA), the protein pellet was washed twice with 800 µl acetone and air-dried. Protein samples were analysed by SDS-PAGE analysis either directly after TCA precipitation or after immunoprecipitation as described[16] with polyclonal antibodies directed to VHH's. Radiolabelled proteins were visualised by phosphor imaging using a personal FX Typhoon scanner (Bio-Rad).

Electron microscopy

Samples from fed-batch fermentations on ethanol and glucose were analysed by transmission electron microscopy as described before[17] with minor adaptations to the protocol: the freeze-substitution medium was 0.1% uranyl acetate in acetone and the immunolabeling was performed with 250x diluted purified rabbit anti-llama IgG K208 and 10x diluted goat anti-rabbit antibodies conjugated with 10 nm gold particles.

Results

Sample characteristics

Ethanol and glucose fed-batch fermentations were performed with *Saccharomyces cerevisiae* strains producing the variable domain of a Camelid heavy chain antibody[18] VHH-BC15 and the antifreeze peptide AFP[19], described before[1,8]. To illustrate the characteristics of these cultivations, the VHH-BC15 fermentation profile is shown in figure 1.

To understand the impact of ethanol fed-batch fermentation on heterologous protein production compared to a glucose fed-batch process, we compared the three different conditions (G4, E1 and E4) with G1, which is most related to the basic conditions described in literature. The comparison of the response ratios can be used for analysing the impact of the reduction in growth rate (G4 vs G1), the difference in carbon source and ethanol stress (E1 vs G1), externally induced stress by a mild accumulation of ethanol in the broth and oxygen

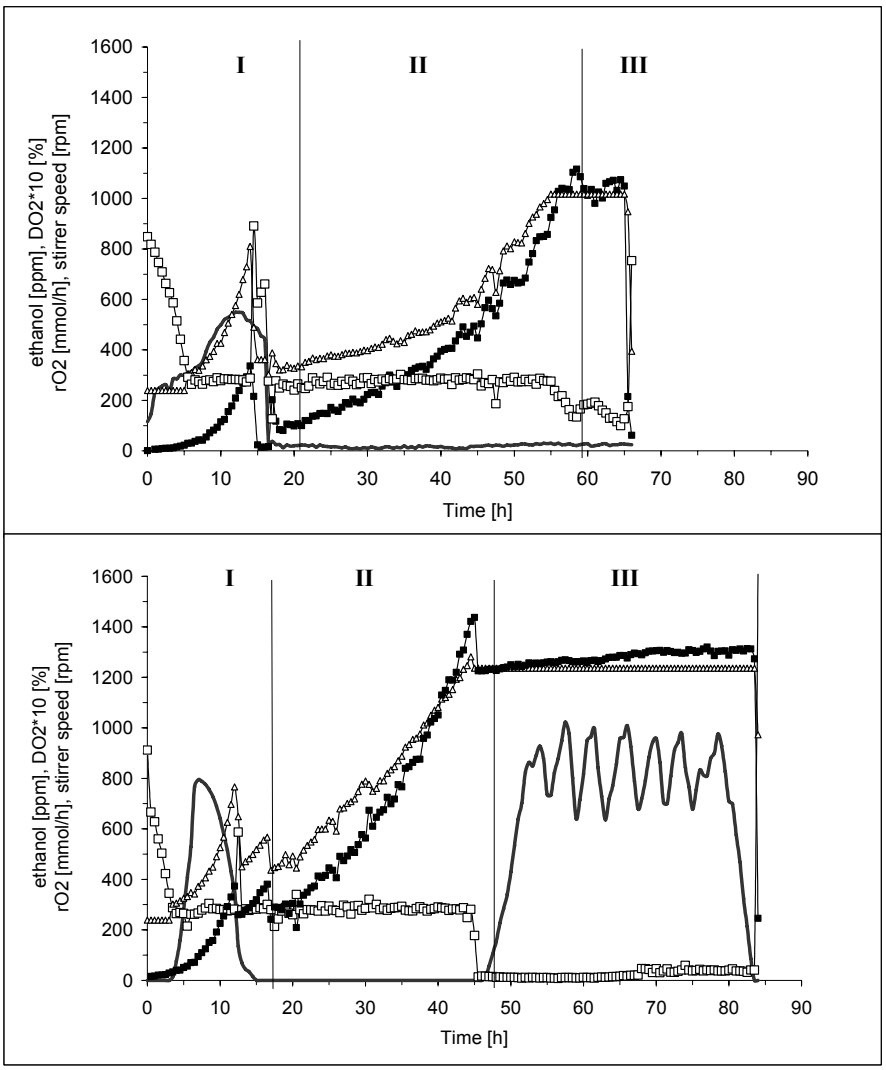


Figure 1.

Fermentation profiles of glucose (A) and ethanol (B) fed-batch fermentations with *S. cerevisiae* producing VHH-BC15.

Ethanol ppm offgas (▬), DO_2*10 % (–□–), rO_2 mmol/h (–■–), Stirrer speed rpm (–△–).

I: Batch phase on glucose. **II**: exponential feed phase on glucose or ethanol. **III**: Decline phase with linear glucose feed and decreased DO_2 or ethanol accumulation and oxygen limitation.

van de Laar *et al*, Chapter 2, this thesis[1]

limitation in combination with ethanol as carbon source and a decreasing growth rate (E4 vs G1).

In table I an overview is given of the sample characteristics of the ethanol and glucose fed-batch fermentations. The G1 and E1 samples were taken from a carbon source limited pseudo steady state after 24 hours, 15 hours after the start of the exponential feed at a growth rate of 0.06 h^{-1}. The E4 sample was taken in the decline phase at approximately 68h cultivation, 22 hours after the start of ethanol accumulation, where oxygen was limiting the culture to a growth rate of 0.03 h^{-1}. The G4 sample was taken also in the decline phase at a

growth rate of 0.03 h^{-1}, 10 hours after the maximum rO_2 was reached. The DO_2 was maintained above 10% to maintain a glucose limited culture.

Table I.

Key characteristics of the samples for DNA microarray analysis. Two fed-batch fermentations with *S. cerevisiae* CEN.PK102-3Agal1 producing VHH-BC15 and CEN.PK338 producing AFP were performed, one on glucose and one with ethanol as sole carbon source. Samples were taken during early cultivation (exponential growth) phase and in the final phase (decline) of cultivation under decreasing growth rate.

	Glucose		Ethanol	
	G1	G4	E1	E4
Ferm. time VHH-BC15	24 h	65 h	26 h	67 h
AFP	27h	67 h	28 h	69 h
Biomass VHH-BC15	18 g/l	101 g/l	22 g/l	78 g/l
AFP	20 g/l	95 g/l	16 g/l	70 g/l
Growth rate	0.06 h^{-1}	0.03 h^{-1}	0.06 h^{-1}	0.03 h^{-1}
DO_2	30%	15%	30%	0%
Fermentation phase	exponential	decline	exponential	decline
Carbon source	limited	limited	limited	1%v/v accumulation

Functional analysis of DNA microarray data sets

The microarray data set of the VHH-BC15 fermentations on glucose and ethanol was analysed before to study the cell status in ethanol cultivation[8], based only on high response ratios (^{2}Log ratio ≤ -1 and ≥ 1) and a high Z-score in the gene ontology annotation (GO).

The response ratios in general are however low. Therefore, to study the less apparent responses a general trend analysis of GO annotations was performed. By this, the gene response level itself is not studied but GO-groups are analysed for general up regulation, neutral response or down regulation. With this functionality analysis it is possible to qualitatively select critical aspects in the cell physiology as a result of ethanol cultivation (E1 and E4), decreasing growth rate (E4 and G4) or a combination of these two (E4).

Here, the influence of the ethanol effect on heterologous protein production was investigated by analysing every step of the intracellular secretion process. This was done by listing applicable GO annotations of the microarray data in the ethanol and glucose data sets for both AFP and VHH-BC15. In figure 2 (page 122) an overview is shown of the different steps and functions in the secretion process. In table II the list of GO annotated groups is presented with the responding notation in the model. In each group the percentage of up regulated (ratio > 0.2), down regulated (ratio < -0.2) genes and genes that have a neutral response in that GO-annotation group are shown.

Table II-A.

Results of functional analysis of secretory pathway according to gene ontology response. Arranged by the location and notation in the secretory model as shown in figure 2. Percentages are calculated as the number of genes per GO group to be up regulated (red), down regulated (green) or neutral (yellow). The highest general response is accentuated by the darker colour.

			E1 vs G1			E4 vs G1			G4 vs G1		
General location	**Model**	**GO annotation**	**UP**	**Neutral**	**DOWN**	**UP**	**Neutral**	**DOWN**	**UP**	**Neutral**	**DOWN**
Ribosomes	B	cytosolic large ribosomal subunit (-sensu eukarya)	0%	13%	87%	2%	17%	81%	4%	25%	71%
Ribosomes	B	cytosolic ribosome (sensu eukarya)	29%	29%	43%	0%	43%	57%	29%	43%	29%
Ribosomes	B	cytosolic small ribosomal subunit (-sensu eukarya)	2%	22%	77%	3%	30%	67%	5%	38%	57%
Protein metabolism	B	protein biosynthesis	7%	7%	86%	10%	29%	62%	12%	26%	62%
Ribosomes	B	translational initiation	6%	16%	78%	3%	25%	72%	13%	44%	44%
Ribosomes	B	tranlational elongation	7%	27%	67%	7%	33%	60%	7%	43%	50%
Ribosomes	B	translational termination	0%	0%	100%	33%	0%	67%	33%	0%	67%
Ribosomes	B	nascent polypeptide association	33%	0%	67%	0%	0%	100%	0%	0%	100%
Ribosomes	B	regulation of translation	60%	20%	20%	60%	40%	0%	60%	40%	0%
Ribosomes	B	negative regulation of translation	0%	0%	100%	0%	0%	100%	0%	0%	100%
Ribosomes	B	regulation of translational fidelity	0%	0%	100%	0%	17%	83%	17%	0%	83%
Ribosomes	B	regulation of translational initiation	0%	60%	40%	0%	60%	40%	20%	40%	40%
Ribosomes	B	regulation of translation elongation	0%	67%	33%	0%	67%	33%	33%	33%	33%
Ribosomes	B	regulation of translational termination	0%	67%	33%	0%	100%	0%	0%	67%	33%
ER	C	protein-ER targeting	0%	44%	56%	11%	0%	89%	11%	0%	89%
ER	C	co-translational membrane targeting	0%	100%	0%	0%	100%	0%	0%	67%	33%
ER	C	srp-dep, co-transl. membr.targ. translocation	50%	20%	30%	33%	33%	33%	30%	40%	30%
ER	C	srp-dep, co-transl. membr. targ, sig.seq. recognition	0%	0%	100%	0%	0%	100%	0%	0%	100%
ER	C	protein signal sequence binding	0%	71%	29%	14%	14%	71%	14%	14%	71%
ER	C	signal peptide processing	50%	50%	0%	0%	100%	0%	0%	25%	75%
ER Structural	C	translocon	25%	50%	25%	0%	100%	0%	0%	75%	25%
ER Structural	C	signal peptidase complex	50%	50%	0%	0%	100%	0%	0%	25%	75%
ER Structural	C	signal recognition particle	0%	50%	50%	0%	0%	100%	0%	0%	100%
ER Structural	C	signal receptor particle receptor	0%	0%	100%	0%	0%	100%	0%	0%	100%
ER Structural	D	endoplasmatisch reticulum	37%	36%	27%	30%	37%	33%	33%	31%	36%
ER Structural	D	ER lumen	40%	0%	60%	20%	80%	0%	20%	60%	20%
ER Structural	D	ER membrane	41%	29%	30%	37%	26%	37%	30%	44%	26%
ER Structural	D	ER membrane intrinsic protein	24%	67%	10%	24%	29%	48%	10%	43%	48%
ER Structural	D	ER receptor	25%	50%	25%	50%	25%	25%	0%	25%	75%
ER	D	hdel receptor	100%	0%	0%	100%	0%	0%	0%	0%	100%
ER	C/D	protein-ER retention	67%	0%	33%	67%	0%	33%	11%	0%	89%
ER	D	ER organization and biogenesis	100%	0%	0%	100%	0%	0%	0%	100%	0%
Protein metabolism	E	protein disulfide isomerase	40%	40%	20%	60%	40%	0%	20%	40%	40%
Protein metabolism	E	protein folding	47%	40%	14%	28%	26%	47%	30%	30%	40%
Protein metabolism	G/H	ER-associated protein catabolism	25%	63%	13%	13%	38%	50%	13%	50%	38%
Protein metabolism	G	protein degradation tagging	100%	0%	0%	50%	0%	50%	50%	0%	50%
Ubiquitin	G	ubiquitin-dependent protein catabolism	77%	19%	4%	35%	36%	29%	17%	39%	43%
Ubiquitin	G	ubiquitin cycle	100%	0%	0%	0%	50%	50%	0%	50%	50%
Ubiquitin	G	protein ubquination	100%	0%	0%	33%	67%	0%	33%	0%	67%
Ubiquitin	G	protein mono and poly ubiquination	63%	26%	11%	58%	21%	21%	32%	42%	26%
Ubiquitin	G	ubiquitin-specific protease	59%	29%	12%	41%	35%	24%	29%	29%	41%
Ubiquitin	G	ubiquitin activating enzyme	100%	0%	0%	0%	50%	50%	0%	50%	50%
Ubiquitin	G	ubiquitin conjugating enzyme	43%	43%	14%	36%	36%	29%	29%	29%	43%
Ubiquitin	G	ubiquitin-like conjugating enzyme	50%	50%	0%	100%	0%	0%	50%	50%	0%
Ubiquitin	G	ubiquitin ligase complex	67%	33%	0%	67%	33%	0%	33%	33%	33%
Ubiquitin	G	ubiquitin-protein ligase	75%	22%	3%	66%	19%	16%	28%	47%	25%
Proteolysis	H	proteasome endopeptidase	97%	3%	0%	20%	70%	10%	13%	37%	50%
Proteolysis	H	proteasome structural	92%	2%	6%	31%	56%	13%	21%	38%	40%
ER	I1	ER to golgi transport	18%	48%	34%	23%	22%	55%	15%	23%	62%
ER	I2	retrograde (golgi to ER) transport	29%	29%	43%	14%	29%	57%	29%	0%	71%
Golgi	J	protein-golgi retention and targeting	70%	20%	10%	0%	60%	40%	30%	60%	10%
Golgi	J	intra-golgi transport	40%	35%	25%	25%	15%	60%	10%	35%	55%
Golgi	J	retrograde (vesicle recycling within golgi) transport	25%	75%	0%	25%	15%	50%	0%	50%	50%

Table II-B.

Second part of functional analysis results as described in table II-A.

General location	Model	GO annotation	E1 vs G1			E4 vs G1			G4 vs G1		
			UP	Neutral	DOWN	UP	Neutral	DOWN	UP	Neutral	DOWN
Golgi Structural	J	golgi organization and biogenesis	0%	100%	0%	0%	100%	0%	0%	100%	0%
Golgi Structural	J	golgi apparatus	38%	32%	29%	32%	44%	24%	15%	44%	41%
Golgi Structural	J	golgi cis-face	17%	58%	25%	25%	25%	50%	9%	36%	55%
Golgi Structural	J	golgi membrane	64%	36%	0%	45%	55%	0%	55%	36%	9%
Golgi Structural	J	integral to golgi membrane	40%	20%	40%	20%	20%	60%	0%	40%	60%
Golgi Structural	J	golgi cis cisterna	0%	50%	50%	25%	0%	75%	0%	25%	75%
Golgi Structural	J	golgi trans cisterna	67%	33%	0%	33%	33%	33%	0%	67%	33%
Golgi Structural	J	golgi trans-face	63%	13%	25%	63%	13%	25%	13%	38%	50%
Golgi Structural	J	golgi transport complex	50%	50%	0%	25%	25%	50%	13%	50%	38%
Golgi Structural	J	golgi vesicle	17%	17%	67%	17%	17%	67%	0%	17%	83%
Golgi Structural	J	trans-golgi network transport vesicle	100%	0%	0%	50%	50%	0%	50%	0%	50%
Golgi	K/L1	post golgi transport	67%	0%	33%	67%	0%	33%	67%	0%	33%
Golgi	K	golgi to plasma membrane transport	68%	26%	5%	26%	42%	32%	21%	47%	32%
Exocytosis secretion	K	exocytose	50%	44%	6%	13%	50%	38%	38%	19%	44%
Exocytosis secretion	K	non selective vesicle exocytosis	100%	0%	0%	50%	0%	50%	0%	50%	50%
Exocytosis secretion	I1/L1	non selective vesicle docking	62%	31%	8%	23%	38%	38%	0%	62%	38%
Exocytosis secretion	I1/L1	non selective vesicle fusion	67%	27%	7%	20%	40%	40%	7%	47%	47%
Exocytosis secretion	K	protein secretion	71%	0%	29%	43%	43%	14%	29%	43%	29%
Exocytosis secretion	K	secretory pathway	67%	33%	0%	56%	33%	11%	33%	44%	22%
Exocytosis secretion	K	peptide pheromone export	100%	0%	0%	100%	0%	0%	100%	0%	0%
Golgi	L1	golgi to endosome transport	67%	22%	11%	67%	11%	22%	44%	22%	33%
Golgi	L1	retrograde (endosome to golgi) transport	100%	0%	0%	17%	67%	17%	17%	50%	33%
Golgi	L1	golgi to vacuole transport	56%	33%	11%	39%	33%	28%	28%	56%	17%
Endosome	L1	endosome transport	100%	0%	0%	100%	0%	0%	100%	0%	0%
Endosome	L1	endosome to lysosome transport	100%	0%	0%	0%	0%	100%	0%	100%	0%
Endosome	L1	late endosome to vacuole transport	77%	15%	8%	46%	31%	23%	23%	69%	8%
Endosome Structural	L	endosome	79%	13%	8%	29%	50%	21%	30%	52%	17%
Endosome Structural	L	early endosome	67%	33%	0%	33%	33%	33%	0%	67%	33%
Endosome Structural	L	late endosome	78%	22%	0%	56%	33%	11%	44%	33%	22%
Vacuole	M	protein vacuolar targeting	75%	18%	7%	50%	36%	14%	24%	50%	26%
Vacuole	M	vacuolar protein processing/maturation	33%	67%	0%	33%	67%	0%	33%	67%	0%
Vacuole	M	vacuole organization and biogenesis	100%	0%	0%	71%	14%	14%	29%	71%	0%
Vacuole	M	vacuolar transport	57%	43%	0%	29%	57%	14%	29%	29%	43%
Vacuole	M	vacuolar acidification	53%	37%	11%	16%	32%	53%	16%	53%	32%
Vacuole	M	vacuolar protein catabolism	100%	0%	0%	57%	0%	43%	14%	29%	57%
Vacuole	M	vacuole fusion (non-autophagic)	71%	29%	0%	57%	29%	14%	29%	43%	29%
Vacuole	M	homotypic vacuole fusion (non-autophagic)	86%	14%	0%	43%	29%	29%	43%	43%	14%
Vacuole	M	vacuole inheritance	85%	0%	15%	31%	38%	31%	38%	23%	38%
Vacuole	M	polyphosphate metabolism	100%	0%	0%	33%	33%	33%	33%	67%	0%
Vacuole Structural	M	vacuole	80%	0%	20%	0%	40%	60%	0%	40%	60%
Vacuole Structural	M	vacuole (sensu fungi)	86%	9%	5%	55%	23%	23%	27%	41%	32%
Vacuole Structural	M	vacuolar membrane (sensu fungi) / vacuolar lumen	62%	33%	5%	38%	43%	19%	29%	48%	24%
Vacuole Structural	M	vacuolar membrane	77%	18%	5%	55%	27%	18%	23%	59%	18%
Vacuole Structural	M	extrinsic to vacuolar membrane	100%	0%	0%	0%	100%	0%	0%	100%	0%
Vacuole Structural	M	hydrogen transporting atpase vo domain	83%	17%	0%	33%	33%	33%	17%	50%	33%
Vacuole Structural	M	hydrogen transporting atpase vi domain	30%	60%	10%	0%	40%	60%	0%	70%	30%
Autophagy	N	Autophagy	74%	23%	3%	61%	39%	0%	42%	58%	0%
Proteolysis		protein catabolism	57%	14%	29%	71%	0%	29%	43%	29%	29%
Proteolysis		proteolysis and peptidolysis	47%	26%	26%	53%	16%	32%	26%	32%	42%
Proteolysis		regulation of proteolysis and peptidolysis	100%	0%	0%	50%	50%	0%	50%	0%	0%
Proteolysis		peptidases	70%	11%	20%	47%	23%	30%	38%	23%	38%
Stress and UPR	F	response to unfolded protein	40%	40%	20%	60%	10%	30%	30%	50%	20%
Stress and UPR		response to oxidative stress	79%	7%	14%	41%	41%	17%	28%	38%	34%
REDOX		regulation of redox homeostasis	80%	0%	20%	60%	30%	10%	40%	30%	30%
REDOX		nadh dehydrogenase	100%	0%	0%	100%	0%	0%	32%	50%	18%
REDOX		glutathion and thioredoxin peroxidase	77%	0%	23%	62%	31%	8%	31%	46%	23%
REDOX		thiol-disulfide exchange intermediate	75%	0%	25%	50%	38%	13%	38%	13%	50%
REDOX		oxygen and reactive oxygen species metabolism	100%	0%	0%	67%	0%	33%	33%	67%	0%

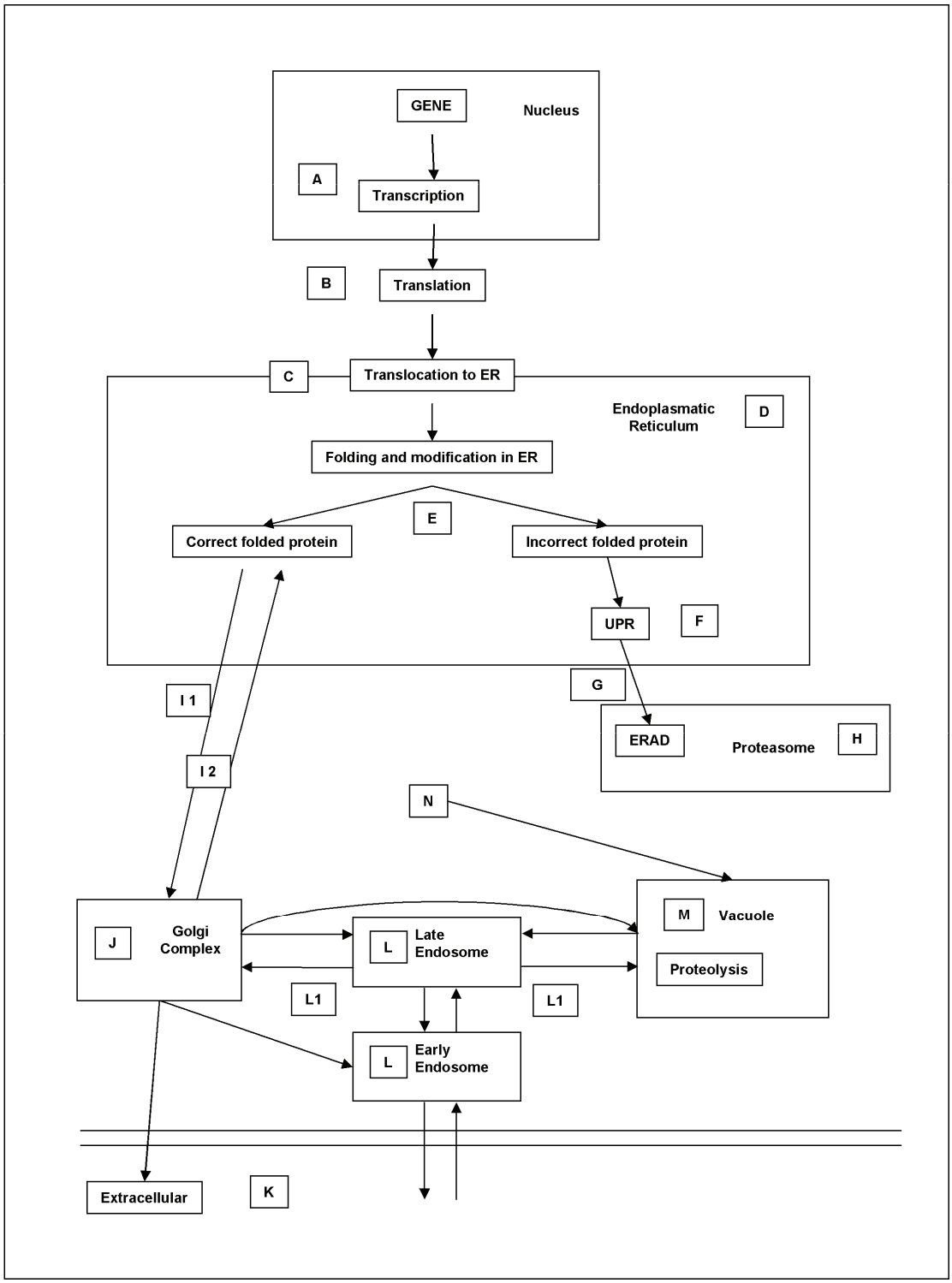


Figure 2.

Protein secretion model. Notations of the several aspects are further described in table II for functional analysis according to GO annotation.

Analyses of the secretion pathway

1. Specific heterologous protein production

The specific production of VHH-BC15 and AFP in glucose and ethanol fermentations is shown in figure 3. It shows that the production increases after induction starts and reaches a

maximum after approximately 40 hours, with a higher production on ethanol than in glucose cultivation. In glucose cultivations the production than decreases in the decline phase under a lowering growth rate. On ethanol however this specific production further increases under a similar decreasing growth rate.

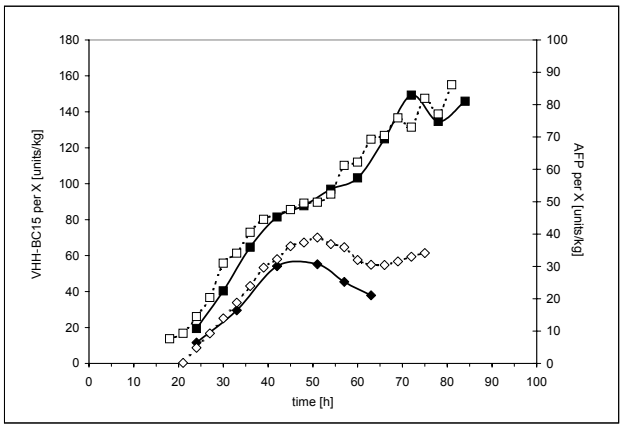


Figure 3.
Specific production (P/X) in fed-batch fermentations with VHH-BC15 (▬) and AFP (▬ ▬) on glucose (◇) and ethanol (□). On the left axis the product per biomass levels of the VHH is shown, on the right axis those of AFP on smaller scale as its inherent production is lower than VHH-BC15. Both productions on ethanol are higher than glucose in a similar ratio. In the final phase of cultivation the productivity or secretion efficiency on ethanol remains increasing while on glucose it decreases.
van de Laar *et al*, Chapter 2, this thesis[1]

2. *Heterologous gene transcription*

The first step in production is the transcription of the heterologous gene after induction of the *GAL7* promoter by galactose. This induction and genes form the galactose metabolic pathway are sensitive to glucose repression[20,21].

Although the glucose concentration in the glucose limited fed-batch fermentation approaches zero, it could still have a repressive effect[22,23]. Indeed on ethanol compared to glucose all genes of the galactose metabolism are up regulated, possibly by the absence of glucose repression (figure 4). However, also in the decline phase of glucose cultivation (G4) the galactose genes are up regulated because there hardly any residual glucose is present. Surprisingly, the *GAL4* response ratios are neutral under all cultivation conditions.

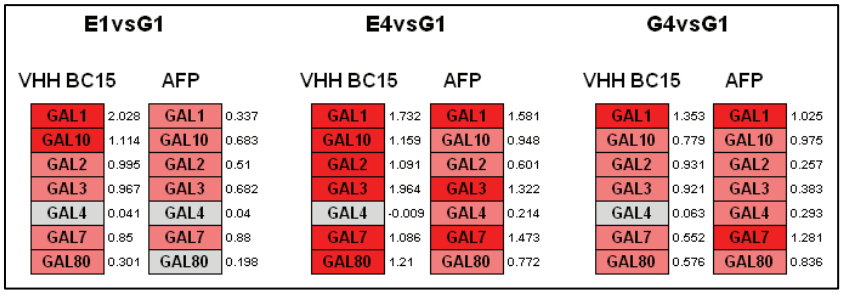


Figure 4.
Expression ratios of genes in the GO groups of “galactose metabolism” compared to standard glucose cultivation (G1) for both the VHH-BC15 and the AFP data set. High up regulation in dark red, up regulation in light red. Grey: no criteria met. All genes are high up regulated on ethanol, but also under lower growth rate (E4 and G4), even under glucose growth. Gal4 however seems neutral or close to neutral under all conditions.

In figure 5 the mRNA levels of VHH-BC15 production are shown. It shows that on ethanol the transcript levels increase to a maximum level in the decline phase after 56h. The mRNA levels on glucose increase first to a lower maximum level and then in the decline phase decrease.

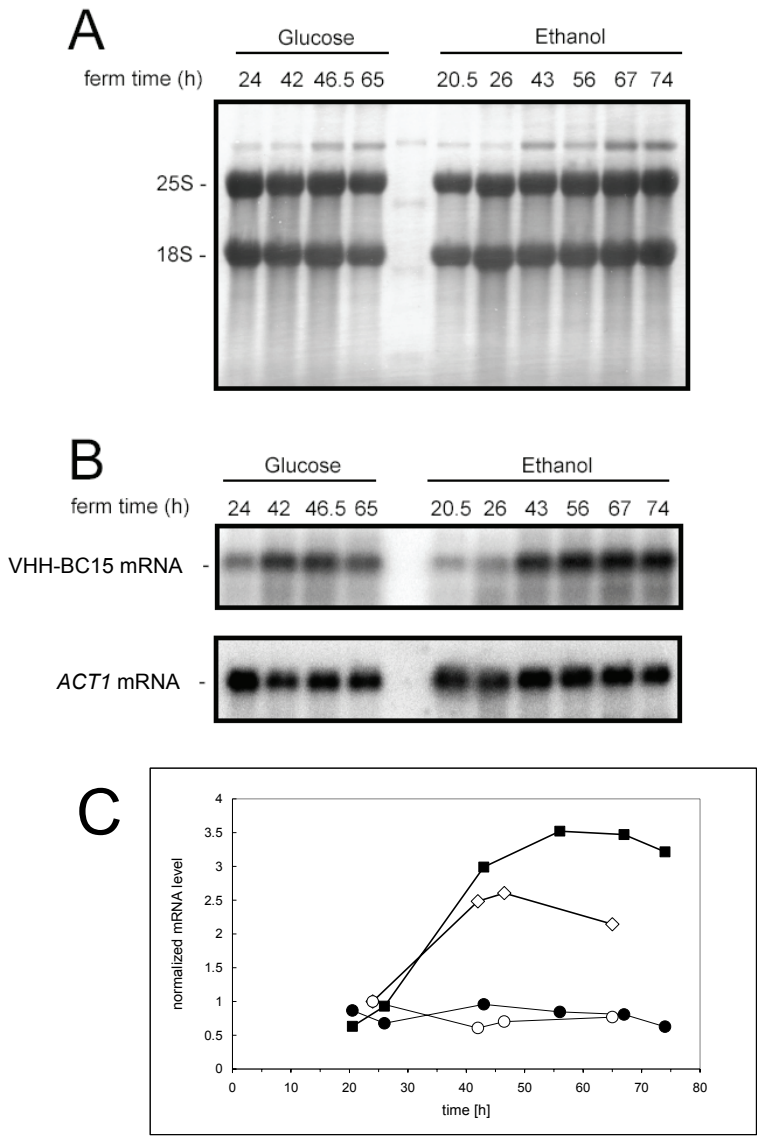


Figure 5.

mRNA analysis of VHH-BC15 samples from ethanol and glucose fed-batch fermentations.

A: Ribosomal bands by methylene blue colouring

B: mRNA levels of VHH-BC15 and *ACT1* for normalisation

C: Graph of quantified and normalised mRNA level of VHH-BC15 on glucose (◇) and ethanol (■) and actin on glucose (○) and ethanol (●) in time.

The mRNA levels under glucose cultivation appear to decrease at the end of the fermentation while under ethanol cultivation it remains high.

For comparison, the microarray transcription level of *GAL7*, which is behind the same *GAL7* promoter as the gene encoding the heterologous proteins, is shown in table III.

Table III.
The transcription levels of *GAL7* in VHH-BC15 and AFP DNA microarrays on glucose and ethanol fed-batch fermentations. The transcription level increases in the decline phase of both ethanol and glucose cultivation.

Data set	*GAL7* transcription level			
	Glucose		Ethanol	
	G1	G4	E1	E4
VHH-BC15	1827	2679	3294	3878
AFP	1366	3320	2514	3792

Although there are only two time points to compare, the *GAL7* transcription increases on ethanol (E1) and in the ethanol decline phase (E4) this transcription further increases. In the glucose decline phase (G4) the transcription has also increased but the level is lower than in E4. This transcription profile of the single copy *GAL7* strongly resembles the multicopy heterologous protein and therefore seems to be regulated in a similar way in spite of the integration of the heterologous gene on the rDNA locus.
The mRNA profile of the VHH-BC15 glucose fermentations resembles also the specific production of the heterologous proteins in figure 3. However the specific protein production on ethanol remains increasing which suggests that also other processes than transcriptional regulation are involved in the ethanol effect.

3. Translation

Table II shows that nearly all GO groups related to ribosomes and translation are strongly down regulated in ethanol cultivation (E1) and that the translational regulation is neutral. Slightly less pronounced, the same response trend is visible in the decline phase of both ethanol (E4) and glucose cultivation (G4). Apparently a combined effect of ethanol and decreasing growth rate represses the translational machinery, as was described in previous work[8].

4. Targeting and translocation

The targeting of the nascent protein to the ER is mainly down regulated or neutral in E1vsG1 (table II). However, the GO group of SRP dependent translocation is up regulated in E1. This up regulation is due to the increased response of SSA1-4, which are highly stress responsive HSP70 proteins that are linked to protein folding[24]. Also the signal sequence processing and the signal peptide complex are up regulated on E1vsG1. Their translocational activity is

however on the lumenal side of the ER, where many ER functional genes are not down regulated as described below.
In the ethanol decline phase (E4vsG1) the translocational responses are neutral or negative, more strongly down regulated than in E1. Also in the glucose decline phase these GO-groups are more down regulated. Therefore translocation seems to be more growth rate than ethanol related similar to the translational down regulation, described above.

5. Endoplasmatic reticulum

The large GO group with genes involved in the processes around the organelle structure of the endoplasmatic reticulum is only mildly up regulated on E1vsG1. A similar, but much smaller GO group containing genes solely linked to the lumenal structure of the ER is down regulated.
However, the GO groups that are specifically related to processes and functions within the ER are all up regulated on ethanol compared to glucose. The strongest up regulated functions are the HDEL receptor gene (*ERD2*) and protein-ER retention, which retain ER proteins in the organelle and the organisational genes. The GO groups of protein disulphide isomerases and protein folding are up regulated on ethanol, but less strong.
In the ethanol decline phase (E4vsG1) the ER structural response seems to further neutralise but the processes in the ER are still strongly up regulated. Only the GO group of protein folding is more down regulated in E4 than in E1.
In the glucose decline phase, under decreasing growth rate, the structural ER genes are mostly neutral regulated while the ER processes are now almost all strongly down regulated. Also the GO group of protein folding is down regulated, like in E4. This group contains also other genes than the ones linked to protein folding in the ER, such as a group of CCT-genes related to the cytoskeleton and thus growth rate[13].

6. Protein degradation ERAD

Also strongly induced on E1vsG1 are ubiquitination and proteasome. This is linked to the ER associated degradation (ERAD) a response that is triggered by UPR[25]. The ERAD group itself, however, shows a neutral response.
In the ethanol decline phase (E4) the response decreases but still there is strong up regulation.
In G4vsG1 the level of the ERAD is neutral which indicates that especially ethanol in combination with heterologous protein production induces ERAD and not the declining growth rate.

7. Transport to Golgi complex

The transport of folded proteins to the Golgi for further processing and distribution and the retrograde path is relatively neutral in E1vsG1. The retention and transport inside the Golgi complex however is clearly more up regulated on ethanol than on glucose.

The decreasing growth rate apparently strongly down regulates the activity towards and inside the Golgi complex as these GO-groups are generally more down regulated in E4 and G4.

8. Golgi complex

In E1 vs G1 the Golgi organisation and biosynthesis is neutral, but this GO-group only contains one gene (*YPT10*)[13]. The structural Golgi groups show a dual response: the GO-groups concerning the cis-face of the organelle are generally neutral in response, while those more related to the trans-face show an up regulated response.

In the ethanol decline phase E4 the cis-face regulation remains neutral. The up regulation of the trans face related GO groups becomes less but in general is still present.

In G4, the decreasing growth rate shows a neutral response and down regulation of the structural Golgi groups, both on cis and trance face.

9. Post Golgi

As already seen in the induction of the trans Golgi complex, the transport from the Golgi to the different destinations is heavily up regulated on ethanol. Exocytosis or plasma membrane transport is a function related to secretion and its up regulation can strongly increase the secretion of heterologous proteins.

In the E4 phase this up regulation is somewhat less but it is still very apparent. While in the G4 stage of the fermentation these GO groups are neutral or down regulated, except peptide pheromone transport (*STE6*)[13] that is up regulated. This is probably induced by the low growth rate response that was described earlier[8].

10. Endosomes and Vacuole

Another secretory direction after the Golgi complex is via endosomes to the vacuole. On ethanol (E1) this transport is heavily up regulated. The vacuole itself and vacuolar processes are also strongly up regulated on ethanol. These processes are mainly concerned with protein catabolism but another function such as the vacuolar polyphosphate metabolism, which functions for accumulation of reserves, is also up regulated on ethanol. The same response profile is witnessed for autophagy.

For comparing the proteolytic response of the cell, GO-groups related to general proteolysis is also shown in table II. The expressed proteins are localised throughout the cell, from cytosolic to membrane bound, and all show strong up regulation on ethanol.

Also in the E4 phase these up regulations are evident. The responses are less pronounced than in E1. These results suggest that the endosome to vacuole transport is induced by ethanol.

This is also evident from the neutral response under growth rate decrease in G4.

11. Stress and Redox

The general response to stress, regulated by STRE genes was shown to be induced strongly on ethanol[8]. The unfolded protein response (UPR) however was more neutral and also in this comparative analysis between VHH-BC15 and AFP production in table II, the response is inconclusive. This could be due to the UPR being already up regulated by the heterologous protein production[26] and the E1 phase therefore doesn't show a distinct response. The oxidative stress response however is more strongly up regulated on ethanol than in glucose cultivation.

Related to this oxidative stress, the impact of the change of redox homeostasis in ethanol fermentations can be seen in the corresponding GO-annotations where it is strongly up regulated on ethanol. Also the NADH dehydrogenase related genes *(NDE1* and *NDE2*) are up regulated on ethanol. These genes provide the cytosolic NADH into mitochondrial respiratory chain[27]. The glutathione and thioredoxin peroxidase group and the oxygen and reactive oxygen species (ROS) metabolism are also highly up regulated on ethanol. Also the expression of genes encoding enzymes involved in thiol-disulphide exchange intermediates (*GRX*, *TRX*) is up regulated. Although they are not ER localised they are induced by oxidative stress and ROS and functionally similar to *PDI1* in the cytosol and other organelles[28,29].

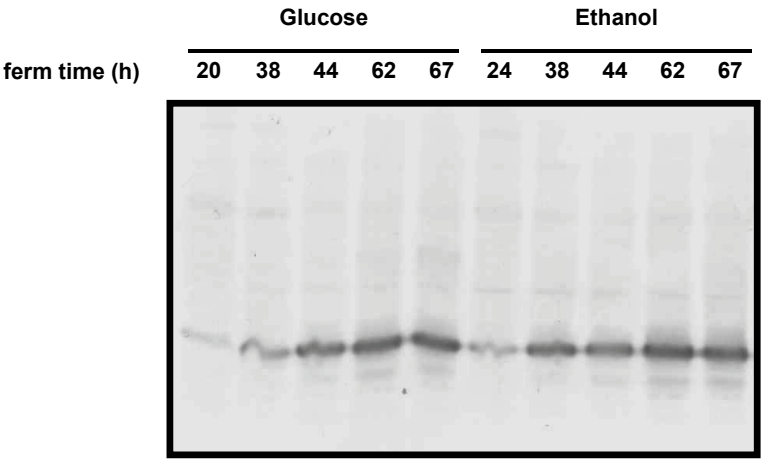
In the ethanol decline phase E4, the UPR seems more strongly up regulated. Apparently under these circumstances the stress further increases, but it doesn't seem to be directly related to a growth rate decrease because the UPR in G4 is neutral.

The oxidative stress and other redox related processes remain all up regulated in E4. In the glucose decline phase however the large majority of these GO-groups are neutral under the growth rate decrease, compared to normal glucose growth.

Internal protein

To verify if the increased secretion and catabolic activity is also evident in the amount of internal heterologous protein a western blot of lysed cells was performed. The results are shown in figure 6. After full induction, the internal protein apparently remains at the same maximum level in both glucose and ethanol conditions. It is therefore not the total amount of protein in the cell but the rate and efficiency that must determine the difference in secretion efficiency of the heterologous protein.

Therefore a pulse chase experiment was performed with VHH-BC15 cells grown in glucose and ethanol fed-batch fermentations. Figure 7 shows that in the ethanol cultivation (E1) the maximum of labelled VHH-BC15 is present after 60 minutes and in the ethanol decline phase this maximum is reached already after 30 minutes. On glucose the highest level of labelled VHH is reached after 60 to 120 minutes and in the decline phase this is also around 60 minutes. The maximum internal levels on both glucose and ethanol seem to be quite similar as earlier shown in figure 6, however the external labelled protein is lower on glucose.



	Glucose					Ethanol				
ferm time (h)	20	38	44	62	67	24	38	44	62	67
total protein (g/l)	191	205	206	195	190	252	195	197	221	215

Figure 6.

Internal VHH-BC15 analysis. Soluble fractions of cracked cells of glucose or ethanol fermentations from different time points were subjected to Western blot analysis. As reference the total protein amount of the concentrated samples was determined by Bradford analysis. In the final phase of the cultivation (>44h) the internal protein does not increase anymore.

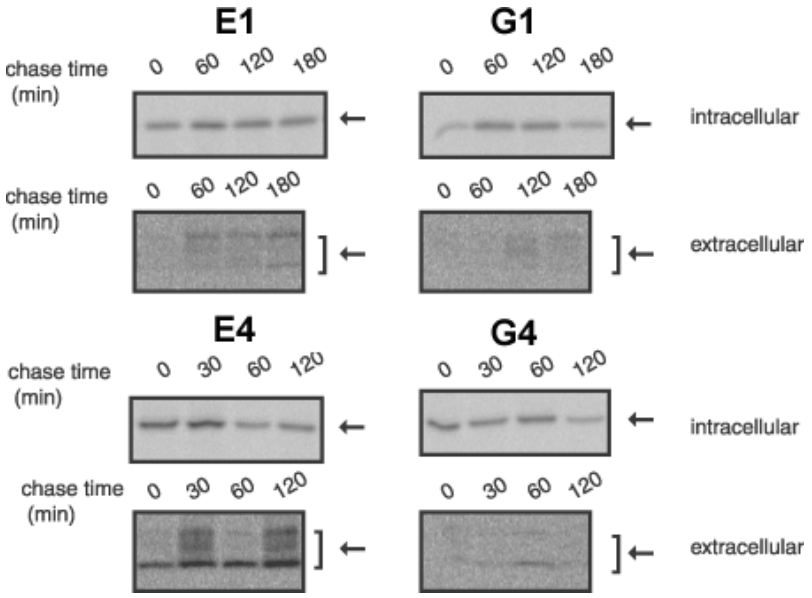


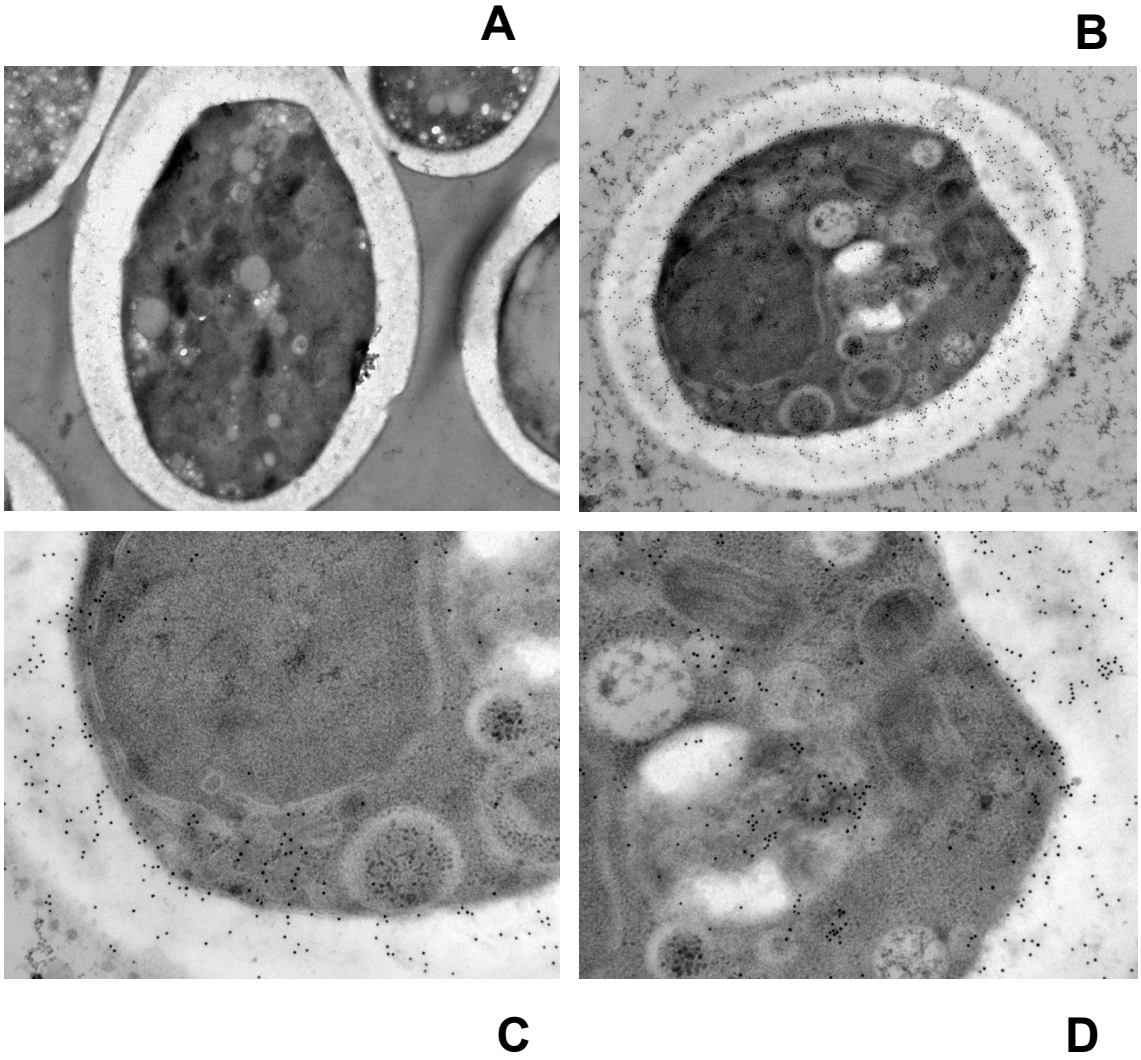
Figure 7.

Secretion kinetics of VHH-BC15 in *S. cerevisiae* grown in fed-batch fermentations.
Cells were labelled with [^{35}S]-Redivue Promix, and chased with unlabelled methionine-cysteine for the indicated time periods. Proteins were analysed either directly (extracellular proteins), or after immunoprecipitation (intracellular proteins) with anti-VHH antibodies, by SDS-PAGE and radiolabelled proteins were visualised with a Phosphorimager. The bracket and the arrow indicate the position of VHH-BC15. Abbreviations: E1, ethanol cultivation; G1, glucose cultivation; E4, ethanol decline phase; G4, glucose decline phase.

To check some of the results of the DNA arrays, like the distribution of the internal heterologous protein, electron microscopy was applied on ethanol and glucose fed-batch grown cells (figure 8) with separate VHH and BiP labelling.

Many cells in both the ethanol and glucose decline phase (E4 and G4) contain thickened cell walls (figure 8A and B), much more than the E1 and G1 cells. These cell walls and the periplasmatic space contain high amounts of apparently accumulated VHH-proteins but also BiP is visible in similar localisation. Especially in the E4 cells, many tubular structures (ER and Golgi complex), vesicles, vacuoles and autophagosomes are seen, all of which show large amounts of immunolabelled VHH (Figure 8C and 8D). It is notable, that this is much more evident in ethanol grown cells than in the G4-cells, although the same effects are visible. It also shows the further diffusion of VHH-proteins through pores in the cell wall to the supernatant fraction.

Figure 8E and 8F show furthermore tubular structures, close to the cell membrane from where an electron dense area and several labelled VHH or BiP-proteins diffuse. This could be an exit site from where proteins are directly secreted from tubular ER or Golgi complex



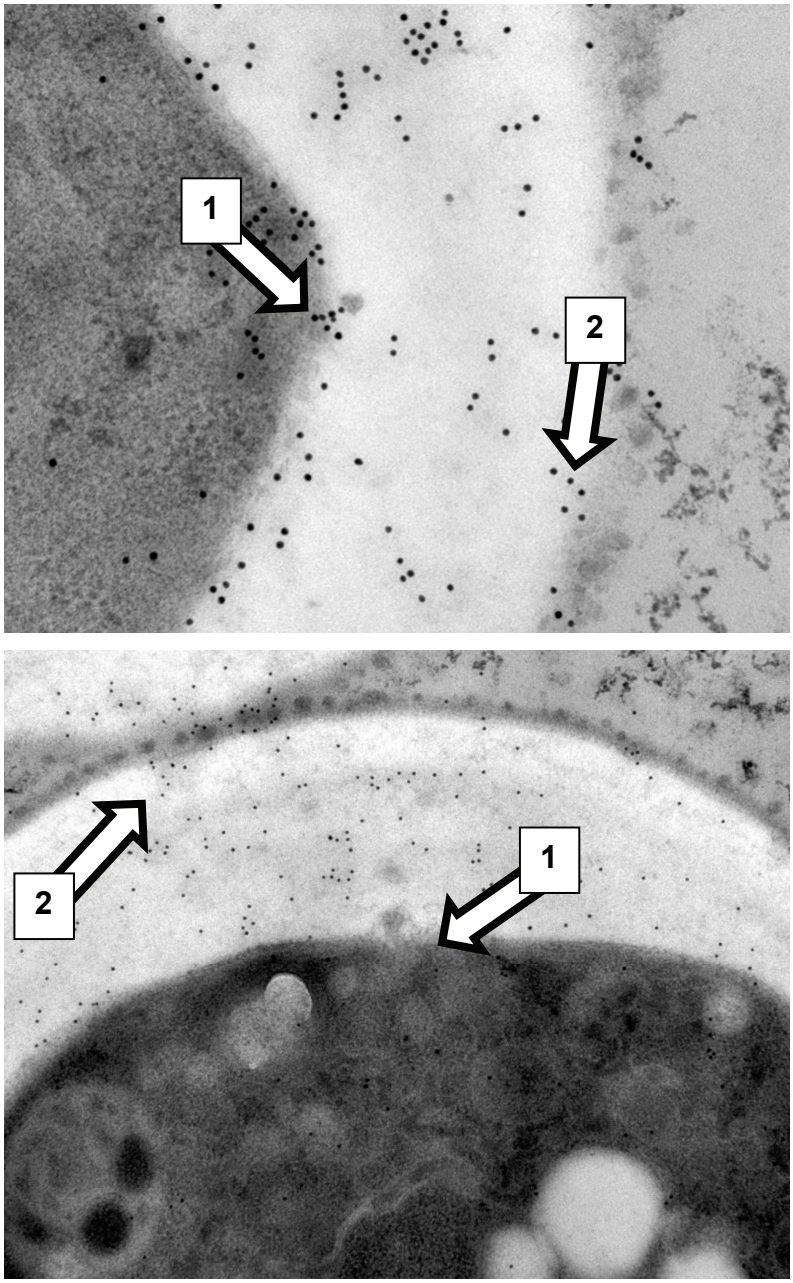


Figure 8.

Electron microscopy analysis of ethanol and glucose grown *S. cerevisiae* strains producing VHH-BC15.

A: Glucose decline phase cells (G4) with immunogold localisation of VHH-BC15 proteins

B: Ethanol decline phase cells (E4) with immunogold localisation of VHH-BC15 proteins

C: Ethanol decline phase (E4), detail picture of figure 8B. VHH localisation in nuclear envelope, endoplasmatic reticulum, vacuole-like vesicles and in cell wall.

D: Ethanol decline phase (E4), detail picture of figure 8B. VHH localisation in vacuoles, autophagosome, vesicles and in cell wall.

E: Ethanol decline phase (E4), detail picture of figure 8D. VHH localisation in tubular vesicular structures and a possible protein exocytosis site (arrow 1), cell wall localisation and leaking from cell wall (arrow 2).

F: Ethanol decline phase (E4). Similar effect as in figure 8E is visible with BiP localisation in tubular vesicular structures and a possible protein exocytosis site (arrow 1), cell wall localisation and leaking from cell wall (arrow 2).

Discussion

The ethanol and glucose fed-batch fermentations with *S. cerevisiae* producing AFP and VHH-BC15 have been analysed in previous studies[1]. The fermentation and specific production profile as well as the DNA array data are similar for both products.
When the heterologous protein production is compared to glucose (G1) it is important to emphasise that the cells are relatively slow growing, producing heterologous proteins under glucose limitation. After the G1 phase the specific protein production first continues and then decreases in the decline phase (G4). On ethanol (E1) the production is higher than on glucose and in the limiting phase (E4) this production continues to increase.

In the previous analysis of the DNA microarray data of the VHH-BC15 experiments the global effect of ethanol cultivation and low growth rate on *S. cerevisiae* was described[8].
The effect of complete glucose absence and ethanol presence changes the metabolism strongly, like described before[3,4,6]. The ethanol passively diffuses into the cell and is subsequently converted to acetaldehyde, which both can accumulate and induces several stress responses like unfolded protein response (UPR), oxidative and general stress response[6-8,30,31]. To cope with ethanol as sole carbon and energy source, the citric acid cycle (TCA) is strongly up regulated[8]. The depletion of carbon from the TCA for anabolic processes seems to induce fatty acid oxidation as an anaplerotic acetyl-CoA source, which is also witnessed in the high up regulation of peroxisome related genes. Side products from this oxidation are aldehydes and peroxides, which have both been described to induce unfolded protein and oxidative stress[31-33]. Although the unfolded protein response (UPR) was less obvious in the analysis of the VHH-BC15 data set, general stress (STRE-regulated) and oxidative stress were very apparent in the ethanol fed-batch cultivation.
Ethanol as carbon source dramatically changes the redox balance in the cell and induces oxidative stress and reactive oxygen species (ROS). For instance, NADPH is no longer yielded in the pentose phosphate pathway but by aldehyde and isocitrate dehydrogenation[4].
The absence of glucose is also an important factor of ethanol cultivation. Many metabolic genes in ethanol degradation, gluconeogenesis and the glyoxylate cycle are regulated under glucose regulated carbon source responsive elements (CSRE)[34] and glucose derepression is witnessed in galactose and glycerol metabolism[20].
Ethanol cultivation furthermore induces the reserve carbohydrate metabolism[8], partly due to stress and a low growth rate. Apart from being a reserve carbon source, trehalose and glycogen are also involved in cell cycling under low growth rates[35] and trehalose has also been described to have a function in maintaining membrane integrity, the protection of proteins against denaturation and in protein folding[36].
Regarding the nitrogen metabolism it was noticed that in the early phase of ethanol cultivation (E1) the amino acid metabolism was repressed while ammonia, as nitrogen source seemed to be directed via glutamate to the replenishment of the citric acid cycle. However under a

further decreasing growth rate (E4), the amino acid metabolism was no longer repressed. This decreasing growth rate was also the cause for the general down regulation of protein synthesis and the ribosomal activity and furthermore, it induced meiotic and sporulation processes.

A very remarkable effect of the ethanol cultivation in the VHH-BC15 analysis is the up regulation of protein catabolic processes. These processes are clearly related to protein secretion, which is therefore discussed in detail based on the results of the experiments performed with the VHH-BC15 and AFP producing strains.

Heterologous gene transcription

The protein production system in these strains contains an rDNA multicopy integration vector, assuring a high copy number[9], with a *GAL7* promoter induced by galactose while the *GAL1* deletion assures abundance of intracellular galactose. Even at low growth rates at least 20 copies of the gene encoding the heterologous product are present (data not shown).

The *GAL7* gene and the genes encoding for the heterologous proteins, both transcribed behind the same promoter show a similar profile, with higher transcript levels on ethanol than on glucose. This implies that some form of glucose repression occurs in the glucose limited cultivations. Although *GAL4*, being strongly repressed by glucose[23], doesn't show any difference between glucose and ethanol. Furthermore many glucose repressible genes are stronger up regulated in the glucose decline phase.

Any glucose repression in a glucose limited system could be explained by the fact that the concentration of glucose has to be extremely low to be fully absent and that the inhibition is a gradual process[37]. Also the reserve carbohydrate metabolism yields monosaccharides that can add to some glucose repression on the Gal genes.

The transcription of the heterologous genes is higher in the decline phase than in the exponential phase, both for E4 compared to E1 and G4 compared to G1. This could be due to the fact that under low growth rates the concentration of any internal glucose is relatively lower than under high growth rates. But more likely the transcription regulation of glucose derepression through Snf1 is a side effect of the cellular response to the low growth rate and possible nutrient limitations. As described before, the regulation of nitrogen catabolite repression, glucose limitation and TOR signalling are highly converged with each other[38-40]. This implies that especially in the decline phase a glucose derepression effect can be witnessed due to a stationary phase or stress response in stead of a glucose concentration.

The similarity between the transcript levels of the single copy *GAL7* gene and the multicopy heterologous protein gene, also implies that the increase of heterologous mRNA on ethanol, or the decrease on glucose in the decline phase can not be due to a change in copy number.

The higher amount of heterologous mRNA in ethanol cultivation versus glucose could also be caused by a change in the mRNA stability or turn over processes under ethanol stress. There is however little known about these processes and the microarray data don't elucidate on this point. It is however described that under general stress[41] and more exactly under ethanol

stress[42] the nuclear export of mRNA is decreased and accumulates in bulk in the nucleus, as a result of Rat8p localisation. How this could result in an increased heterologous protein production is unclear but it shows that ethanol is also effective on the level of mRNA and the nucleus.

In glucose cultivation the mRNA profile of the heterologous gene transcription has a similar profile as the specific protein production level. But on ethanol this is not the case, the specific production remains increasing towards the end while the heterologous mRNA level remains at a maximum or even decreases slightly. For the production efficiency of the heterologous protein the amount of endogenous mRNA should also be taken into account. As the growth rate decreases and as a result of TOR signalling, the endogenous mRNA levels are likely to decrease yielding a higher capacity for heterologous proteins.

Translation

Although the transcription levels of the heterologous gene are much higher on ethanol, the ribosomal and translation machinery for endogenous protein production is strongly down regulated on ethanol and under low growth rate, as also seen in the previous array analysis[8]. This response is a typical stress response to low growth rate and changes in nutrient availability[43] and ascribed to TOR signalling[40,44]. These aspects are evident especially in the decline phase of ethanol cultivation. But as this phase is the most productive phase in heterologous protein production, the capacity of the ribosomal machinery is apparently in excess. Therefore also translation is unlikely to be the limiting factor in secretion efficiency. What is further evident from the results is that processes on the cytosolic side of the ER are down regulated while the lumenal side is up regulated. This also implies that the ethanol effect is more focused on folding than on translation and translocation.

Protein folding

In ethanol cultivation the genes involved in the proliferation and maintenance of the ER structure show a mixed response but in general they are mildly up regulated. The functions within the ER though, show a more uniform, up regulated response on ethanol. One of the main effects of the unfolded protein response (UPR) is induction of several functions within the endoplasmatic reticulum[45,46]. This response however is already induced by the high heterologous protein expression, depending on the efficiency of folding into the tertiary structure determined by its amino acid sequence and less on the carbon source[26]. Therefore the more ER related effects of the UPR in the ethanol fermentation are not as strong as others but it seems that ethanol does have a further up regulating influence on protein folding and modification.

Furthermore, it is evident that a decreasing growth rate like in G4, neutralises and even down regulates structural ER effects and processes like protein folding in general. But under declining growth rates in ethanol cultivation this is much less clear.

The UPR related ERAD regulation itself is not so strongly up regulated but its effectors, ubiquitin cycle and the proteasome are very clearly up regulated on ethanol. Under decreasing growth rates these processes are neutral or down regulated but in ethanol cultivation there is still ubiquitination and protein catabolic activity.
Although the direct UPR is only slightly improved on ethanol, the disulphide bond formation of folding proteins and the breakdown machinery of misfolded proteins is very active on ethanol and not in the decreasing growth phase on glucose. This can have a big impact on the capacity and efficiency of protein folding and secretion especially under lower growth rates where the amount of endogenous protein decreases and the heterologous protein flux can further increase.

Redox effects on protein folding

Ethanol in induces a dramatic metabolic and anabolic change and as a result also the redox balance can change significantly[2,5]. The exact ratios of NAD, NADP and FAD over their reduced counter parts compared to glucose are difficult to predict or measure however, depending on growth rate, cell state and compartmentation. Besides a change in redox balance, ethanol cultivation induces YAP1 regulated oxidative stress[8,47] and reactive oxygen species like peroxides from for example beta-oxidation[8] but also from protein folding processes in the ER[48]. This all strongly influences the glutathione redox buffer (GSH/GSSG) and related systems like the glutaredoxin and thioredoxin system which are necessary for maintaining the redox balance in the cell and for several processes like protein folding and disulphide bond formation[49].
Especially in the endoplasmatic reticulum protein folding is strongly dependent on the redox balance and the availability of flavin adenine dinucleotide (FAD)[48]. The disulphide bridge formation in protein folding is catalysed by the FAD dependent Ero1p with Pdi1p as intermediate. And although the redox buffer (GSH/GSSG) ratio is directly determining the folding efficiency it is related to the process[50] and depending on the cytosolic balance. Therefore the redox state and the thiol formation efficiency in the ER seem to be dependent on the carbon source on the one hand and oxidative stress and ROS on the other.
This relationship seems confirmed by an earlier described experiment[51] with VHH in ethanol and glucose cultivation. VHH-BC6 was produced under acceptable productivities on glucose. When the amino acid sequence of this VHH was extended by short sequences containing an additional cysteine residue on the C-terminus the productivity was almost completely lost on glucose. However when the strain was cultivated on ethanol the productivity was restored almost completely to the earlier production level.

Golgi complex and trans Golgi network

The results show that transport to the Golgi complex is only mildly induced and that the Golgi complex is equally developed under glucose and ethanol conditions. However, the export from the trans side of the Golgi complex is strongly up regulated on ethanol.

In this trans Golgi network (TGN), proteins are sorted and transported to their destination[52]. This can be done via several routes with or without endosomal action towards secretion or to the vacuole[53] (Figure 2). The secretion pathway and the proteolytic pathway are therefore closely related through the trans Golgi network. On ethanol this is reflected in the up regulation of exocytosis, structural endosomes and endosomal transport as well as vacuolar transport and the vacuole itself. This increased transport activity can increase the secretion capacity and efficiency dramatically, through which the heterologous protein secretion can benefit.

When the growth rate decreases in the ethanol cultivation the up regulation of the TGN becomes less strong but is still evident, but again the decrease of endogenous proteins can yield a larger secretion capacity for the heterologous proteins. In the glucose cultivation, the growth rate down regulates the Golgi complex and the TGN becomes more neutral as the cell metabolism becomes less active. It was reported earlier[8] that the declining growth phase on glucose induces much more pheromone and meiosis related gene expression. This could explain why only the peptide pheromone transport is up regulated in ethanol cultivation and in the glucose decline phase.

In general though, for the TGN there seems to be a contrasting dual effect of ethanol and decreased growth rate.

Vacuole, autophagy and proteolysis

As described above the vacuole, vacuole transportation and its catabolic processes are strongly up regulated on ethanol. Also autophagy, proteolysis and ubiquitination are up regulated. These catabolic processes are, as it seems vital to ethanol cultivation. In yeast the bulk of protein catabolism can be processed by proteasomal activity. Larger aggregates, cytosolic macromolecules and even organelles however can be catabolised by autophagy[54]. Under conditions of stress like starvation or adaptation to a change of environment the autophagy pathway is strongly induced to adapt the cell state and to recycle amino acids and lipids[55]. Actually, autophagy is regulated under the TOR signalling pathway, which could explain the high up regulation on both ethanol and decreased growth rate[44,55].

So under ethanol cultivation the catabolic and more specifically proteolytic processes are strongly up regulated, to cope with the stress state, adapt to the environment and turn over of cell material to provide in amino acids and fatty acids for biogenesis. But as the total internal protein doesn't significantly differ between glucose and ethanol cultivation, apparently the increase in capacity and efficiency induces a higher production rate on ethanol.

Protein secretion kinetics

The northern and western blot and the specific production plot are confirmed by the pulse chase experiment, which additionally shows that the difference in secretion is caused by an increased protein flux through the secretion pathway. Unfortunately the data of the pulse chase can not be quantified and therefore remain inconclusive towards the catabolic turn over

of proteins as a major influence on secretion efficiency. By designing more fine tuned experiments in for instance continuous cultures on defined media this could be studied in much more detail.

The electron microscopy analysis has verified the increase of cellular structures like ER, Golgi complex, vesicular transport and moreover autophagosomes and vacuolar organelles in the ethanol decline phase. These secretory and catabolic activities aid in the processing of the increased heterologous protein flux.
An additional unexpected issue of heterologous protein production in yeast cells is the appearance of thickened cell walls in older cultures and the accumulation of VHH and also BiP proteins within. Apparently, older cells that have been producing large amounts of heterologous proteins and have been budding for a long period, accumulate excess protein outside the cell membrane. From this buffer the protein further diffuses through the cell wall into the supernatant fraction. The fact that also BiP is localised outside the ER, where it should be retained or transported back to, can be explained by the fact that it binds to protein aggregates in the ER and due to high flux of heterologous proteins it is co-secreted. Another explanation for this could be that the membranes of ER or Golgi are so strongly proliferated or fluidised that they easily melt with the plasma membrane, thereby loosing BiP. The appearance of the secreting tubular structures in figure 8E and 8F could subscribe this.
It is not possible to comment on the kinetics of the accumulation and transport of the VHH in the cell wall structure, but it is very well possible that ethanol has an additional effect on secretion here, by acting on the cell wall permeability, which will be studied in future research.

Conclusion

To reduce the complexity of the various steps from transcription until secretion of heterologous proteins and to place these steps in the right context, it is appropriate to dissect these processes into those related to the heterologous protein and endogenous proteins that reach their destination via the ER. Moreover a number of processes have both an influence on endogenous proteins that remain in the cytoplasm. Also these proteins contribute to the overall context of the secretion process. In particular this is the case for cytoplasmic proteins that are modified by ethanol or its metabolites, including ROS. These modifications often result in proteins with improper folding and are prone to degradations by one of the three main routes for that. The overall process can be described easily in an in- and out put model of a black box. The data for output (g product/g biomass), corresponding for E1, E4 and G4 (all relative to G1) are clear: slightly up for E1, strongly up for E4 and slightly down for G4. However the black box can be dissected into a number of smaller black boxes, for which often in- and output can be estimated on basis of our results and for which the various effectors of

the black box are known, at least qualitatively. The summarised effectors on these black boxes are shown in figure 9.

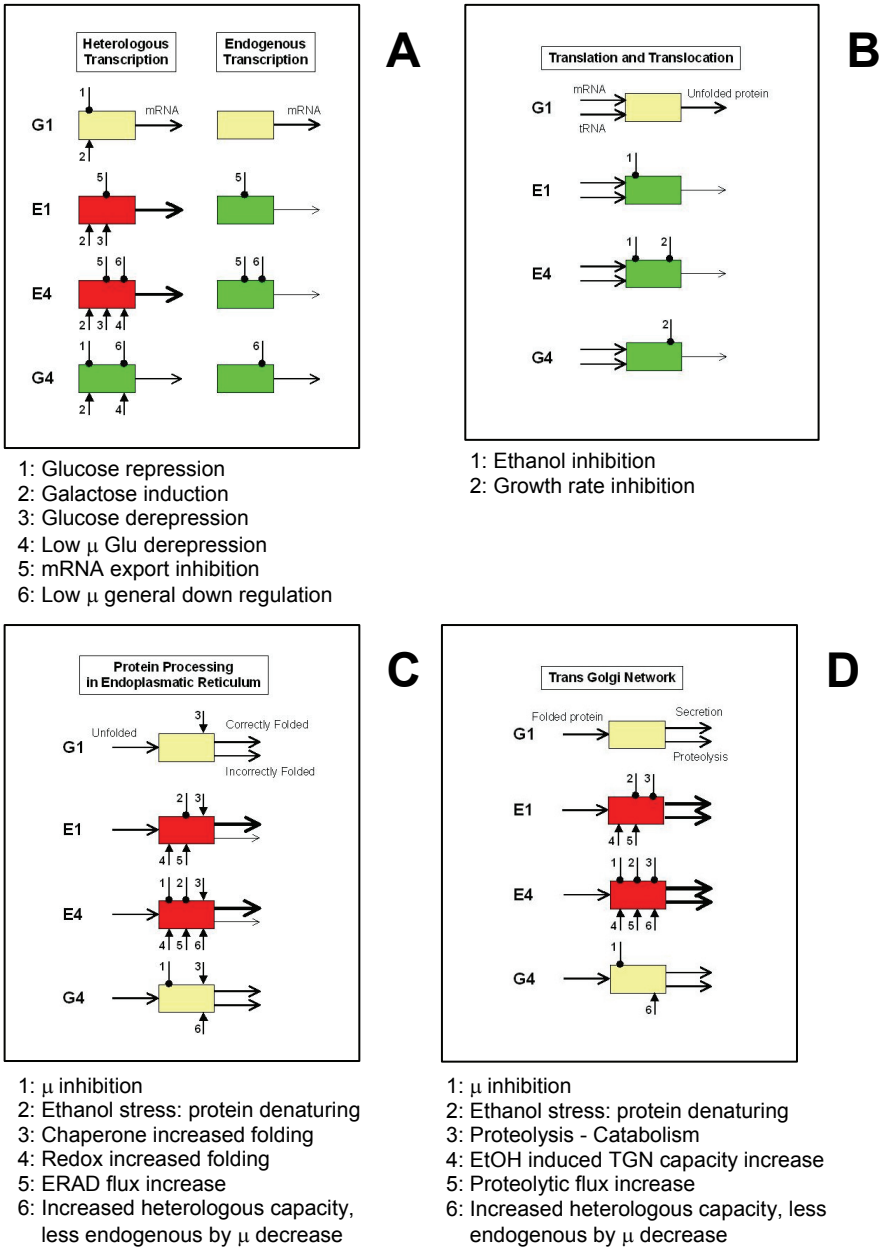


Figure 9.

Black box models and effectors (arrows: positive effect, rounded arrows: negative effect) for several determining steps in the secretion pathway. Indicated by colours are the general regulation of each step, up regulation in red, down regulation in green and a neutral effect in yellow. Under secretion process step the effectors are mentioned.

Subsequently each individual black box is integrated in the overall black box in figure 10, for the two extreme situations: production under glucose exponential growth (G1) and under ethanol decline phase growth (E4).

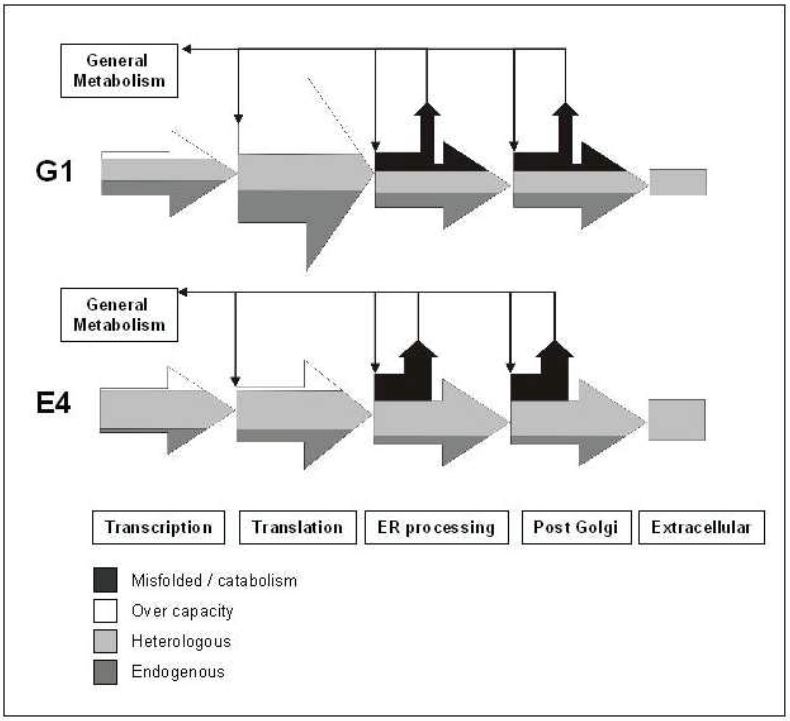


Figure 10.

Integrated black box model for the essential steps in protein production and secretion. The relative amounts of endogenous, heterologous and unfolded proteins are shown in step arrows that are adapted in size to the expected capacity. Small black arrows show the turn over of amino acids and lipids in catabolic processes. For comparison to glucose the ethanol decline phase (E4) was taken, comprising the ethanol metabolic effect, stress and low growth rate. In E4 the transcription step yields more heterologous mRNA, the translational capacity is however decreased, but the ER processing and post Golgi transport are more efficient, yielding more heterologous product.

As a result of the several effectors on ethanol the heterologous transcription capacity increases, while the total translation capacity decreases but can be still sufficiently large to not hinder increased efficiency. As a result of an increase in correct folding, proteasomal activity and lower endogenous protein processing, the flux through the endoplasmatic reticulum can be higher. And a similar reasoning can be made for the trans Golgi network, finally resulting in higher external levels of heterologous proteins. The catabolic turn over vesicles and proteins can be directed to the secretion pathway again or for metabolic and anabolic use in fatty acid beta oxidation, amino acid metabolism and the central carbon and energy system of the citric acid cycle[8].

The effectors and finally the overall ethanol effect seem to be regulated under different signalling pathways but the main one seems to be TOR signalling, which among others influences glucose derepression, autophagy and amino acid metabolism. Apparently ethanol cultivation apart from general, oxidative and unfolded protein stress induces nutritional adaptation and a stationary phase stress that results in a side effect of increased heterologous protein production that can be extremely beneficial for industrial process applications.

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CHAPTER 6

General Discussion

Production optimisation

The production of heterologous proteins is of great importance for industrial applications in fields such as pharmaceutical ingredients and industrial enzymes. But the success of product development is being determined by the final product yield, the costs and the feasibility of the process. If these criteria are not met, potential products become stuck in the pipeline. Therefore the production of heterologous proteins in industrial processes is always under development to make processes more cost effective. Two of the most important ways of optimisation of these processes are strain improvement (biological optimisation) and process improvement (technical optimisation).

Production strains have a number of features in which they can be biologically optimised, varying from promoter system, copy number and stability to signal sequences. Also, secretion enhancement by inducing unfolded protein response genes[1,2] and metabolic engineering to optimise carbon en energy streams towards protein production are powerful tools[3]. But the whole biological system and its regulatory network are extremely complex. For instance the deletion of certain glycosylation genes can induce others to take over. A similar effect can occur with protease deletions, which can induce others to more rapidly degrade the product of interest. Therefore, the biological improvement of production by directed modification and its fine-tuning can be a very time consuming and expensive process. That is why very often random mutagenesis is applied for production enhancement[4]. This can be very successful, but until recently it was a very elaborate and undirected method by which unwanted side effects could appear, also depending on the product application. Now, with the aid of robotics large mutational screening programs can be executed. And through DNA microarrays it is possible to see the mutational changes on a genomic level, which has made random mutagenesis much more transparent.

The technical improvement of heterologous protein production is linked to fermentation improvement and increase in recovery yield and quality. In this thesis we focus however on upstream processes and will therefore only discuss fermentation optimisation. The ultimate goal of technical upstream optimisation is the highest volumetric productivity[5,6], more product per volume per time at the lowest possible cost. For that the optimum parameters have to be determined such as cultivation method, the best growth rate, temperature, pH, oxygen regime, feed profile, inoculum train and medium optimisation. But also process control, medium costs and logistics are very important for the optimal productivity.

One of the most commonly used and extensively studied production systems for heterologous proteins is *Saccharomyces cerevisiae*. In most high yielding processes *S. cerevisia*e is cultivated in large scale fed-batch fermentations[5,6] with glucose or molasses as carbon and energy source. Although much is known about this eukaryotic host strain and its production

processes, still much work is performed on further improving its production system. In this work the improvement of the production of Camelid antibody fragments (VHH) by *Saccharomyces cerevisiae* in fed-batch fermentation was investigated both on biological and technical aspects and we found that the use of ethanol as sole carbon source greatly increases this production[7].

The effects of ethanol on the production efficiency are both biological and technical. It increases the specific production of heterologous proteins especially towards the final phases of fermentation, under ethanol accumulation and decreasing growth rates[8]. Therefore it also makes the production more stress resistant and robust. Furthermore, heterologous proteins that are difficult to produce due to their sequence and conformational properties showed significantly increased production on ethanol. In this chapter the several aspects of biological and technological optimisation of this ethanol based heterologous protein production system are discussed in more detail.

Biological aspects of ethanol cultivation

When *Saccharomyces cerevisiae* is cultivated in an excess of glucose, it produces ethanol as a result of aerobic alcoholic fermentation. This is also known as the short term Crabtree effect[9]. Once the glucose concentration has decreased below a threshold level the yeast will start the consumption of ethanol after a diauxic shift in which the cellular metabolisms changes significantly[10].
Ethanol therefore has a large impact on yeast cultivation and mostly it is regarded as an unfavourable and even toxic side product. It is a less efficient carbon and energy source than glucose and therefore the biomass yield is lower and also growth rate is inhibited[11,12]. In addition, it has been described that ethanol has a strong effect on the plasma membrane by acting on its fluidity[13], permeability[14], transport and ATPase activity[15].

More recently by using DNA microarray techniques much more insight in the effects of ethanol on *S. cerevisiae* have become apparent. An enormous amount of data was generated by many different studies[10,13,16-19], each having its specific characteristics but adding to the general understanding.

Gasch *et al.*[16] described the genomic response of ethanol stress for the first time in a very broad study into many different environmental stress responses in *S. cerevisiae*. For the ethanol response the cultivation was performed in shake flasks with ethanol and compared to glucose batch cultivation. The cultivation on glucose in shake flasks is however under fermentative glucose metabolism which produces ethanol. The excess amount of glucose induces carbon catabolite repression and the specific growth rates vary from low to maximum.

Furthermore the accumulation of ethanol in the cell and in the culture can already induce some of the ethanol effects that remain unnoticed in these comparative studies. For a pure ethanol response compared to glucose cultivation the glucose consumption should be strictly oxidative as in the glucose limited fed-batch fermentations we performed[20], or such as the continuous glucose cultivations of Daran-Lapujade *et al*.[18]. Many of the findings of Gasch *et al*.[16] are however very valuable to the study of the effects of ethanol. For instance, they did not describe the ethanol stress as a separate stress effect but noticed that many different impulses show a large overlap in stress response and gathered them under the Environmental Stress Response (ESR). As the cellular response systems are extremely complex this ESR is probably the best way of grouping stress responses. Furthermore, Gasch *et al*.[16] also described how a stress impulse is dealt with in a cell: the first impulse induces a strong transcriptional response, which increases with the strength of the impulse. Over a longer period this effect is dampened and only small differences in the adapted cell state remain noticeable.

DeRisi *et al*.[10] were one of the first to perform DNA microarrays on *S. cerevisiae* to study the diauxic shift from glucose fermentative to ethanol oxidative metabolism. This again introduces the same limitations to the data analysis for an ethanol response but has given valuable data and verification on the metabolic changes. They closely described the carbon metabolic pathway on glucose and ethanol and the changes between them. They also introduced very useful methods on sampling and RNA isolation and on performing and analysing DNA microarrays[21]. Furthermore they instigated the use of web-based programs and DNA array databases for global comparison of data.

The first to exclusively study the response to ethanol in *S. cerevisiae* were Alexander *et al*. [13]. Again their study was performed in glucose grown shake flasks. The ethanol pulse that was given to these cells was relatively high (7% v/v) and after 30 minutes the cells were harvested for RNA isolation. This makes the ethanol stress a short term, high impulse effect and misses out on slower, lower responses and what happens when cells are exposed to long term mild stress. Their analysis of the microarray data however is extensive and thorough and has shown many new aspects and links to cellular responses. For instance, they showed the induction of genes related to heat shock proteins, reserve carbohydrates and oxidative stress as well as the importance of the energetic changes upon ethanol stress.

Chandler *et al*.[17] extended on the research done by Alexander *et al*.[13] by performing a similar experiment but exposing cells longer (1h and 3h) to high ethanol stress (5%v/v). Actually the cells in these experiments seem to be recovering from a lag-phase, induced by the change of medium and carbon source instead of normal cultivation. The results show little additional information except for a transient response of the cells.

Daran-Lapujade *et al*.[18] has described the true ethanol response compared to glucose oxidation. They performed continuous cultures in mineral carbon limited media under long term ethanol exposure. The absence of any ethanol accumulation in the cultivation makes the stress very mild. The cells are however in a very well defined steady state with a constant growth rate, fully adapted to the ethanol conditions. The analysis of the array data focused on the metabolic response, fluxes and some possible transcription factors regulating this response. Many aspects of their results were used in this work to validate the metabolic responses and conclusions of our microarray data.

The only example of ethanol stress under industrial conditions is the work of James *et al*.[19]. The DNA microarrays were performed on *S. cerevisiae* under very different conditions in a sugar fermenting brewing process. The environment consists of high accumulating ethanol concentrations (up to 8%v/v), anaerobic conditions, low temperature (11°C) and low cell densities. Their results however agree to a large extent with earlier publications and show overlap with our data in peroxisomal up regulation and protein degradation.

Through this work and many other, the knowledge of ethanol stress has increased. It was however unknown what the cell status is during heterologous protein production by *S. cerevisiae* in ethanol fed-batch fermentations and what the relation is to the increase in protein production that we have demonstrated. These processes were performed under very characteristic conditions; different from the previously described DNA array experiments.

In our production strain, the heterologous protein is encoded by a multi copy vector integrated in the ribosomal DNA locus, which results in a high and stable copy number. Furthermore, the gene is placed behind the strong *GAL7* promoter while a *GAL1* deletion assures that galactose is not consumed.

The key characteristics of the fermentation profiles are shown in figure 1a and 1b, for both glucose and ethanol fed-batch fermentations. The ethanol concentration in the beginning of the cultivation is limited while at the end, when the highest specific production occurs the ethanol accumulates to 1%v/v. During this period the growth rate is relatively low and decreasing at the end. Furthermore the medium is rich, containing yeast extract and ammonium and the biomass concentrations are high. In this long cultivation the cells have fully adapted to the environment and show a long-term response. For comparison, samples were analysed from glucose limited fed-batch fermentation, comprising an exponential phase and a decline phase where glucose is still limiting but the growth rate decreases.
A summary of they characteristics of the DNA array samples is shown in table I.

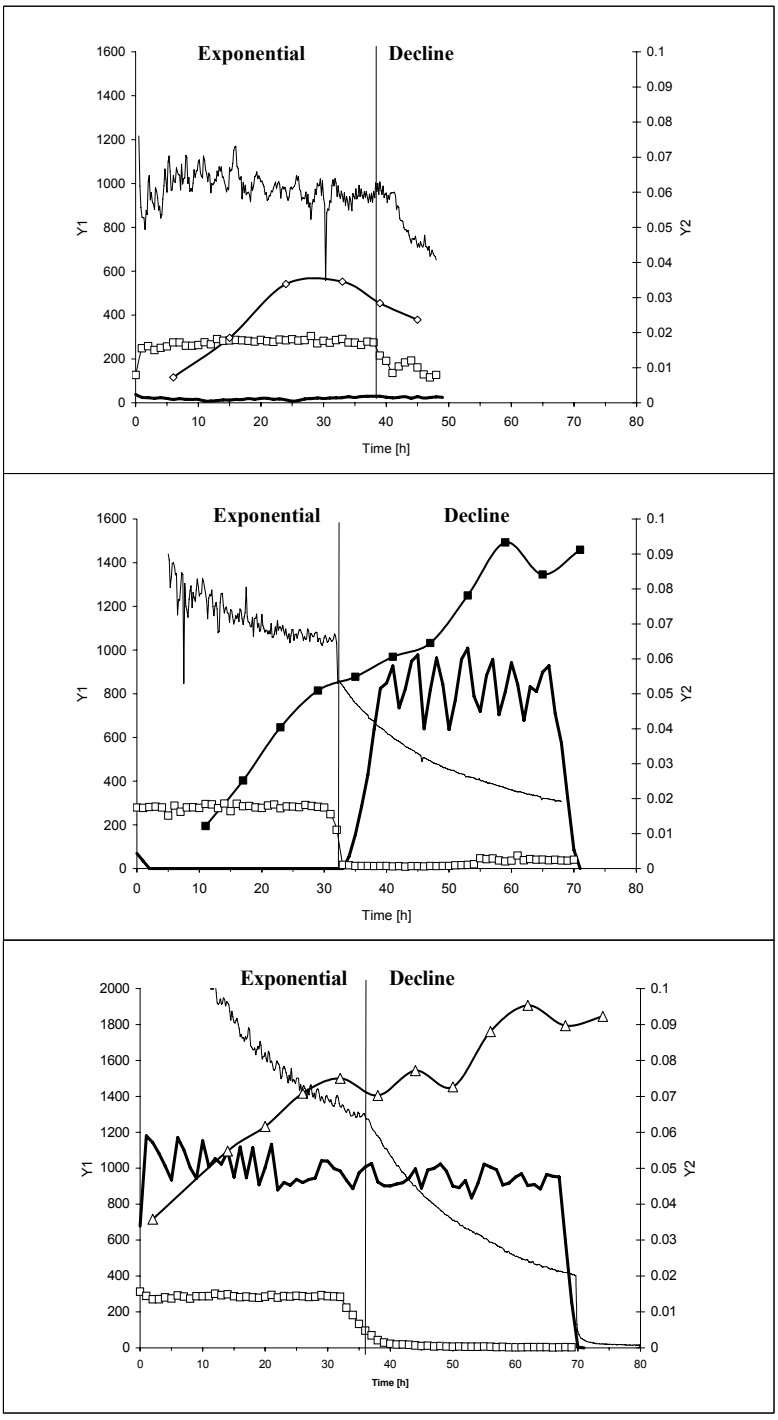

Figure 1.

Feed phases of fed-batch fermentation profiles with *S. cerevisiae* producing VHH-BC15.

Y1 axis: Ethanol ppm offgas (▬), DO_2*10 % (–□–), P/X *10 units/kg (◇ , ■ , △).

Y2 axis: Growth rate h^{-1} (—).

A: Glucose fed-batch fermentation. Exponential feed phase under glucose limitation at a growth rate of 0.06 h^{-1}. Decline phase under glucose limitation and DO_2-concentration of minimal 10%. B: Ethanol fed-batch fermentation. Exponential feed phase under ethanol limitation at a growth rate of 0.06 h^{-1}. Decline phase under ethanol accumulation of approximately 1000 ppm in offgas and a DO_2-concentration close to 0%. C: Ethanol fed-batch fermentation. Feed phase under ethanol accumulation of approximately 1000 ppm in offgas. Decline phase under 1000 ppm ethanol accumulation and a DO_2-concentration close to 0%.

Table I.
Sample characteristics of glucose and ethanol fed-batch fermentations at distinct time points.

	Glucose		Ethanol	
	G1	G4	E1	E4
Fermentation time	24 h	65 h	26 h	67 h
Biomass	18 g/kg	101 g/kg	22 g/kg	78 g/kg
Growth rate	0.06 h^{-1}	0.03 h^{-1}	0.06 h^{-1}	0.03 h^{-1}
DO_2	30%	15%	30%	0%
Fermentation phase	exponential	decline	exponential	decline
Carbon source	limited	limited	limited	1%v/v accumulation

By comparing the different data sets to the standard glucose phase (G1) it was possible to investigate the impact of both ethanol limitation and excess versus glucose limited growth and the influence of a decreasing growth rate. Based on these DNA microarray analyses supported by pulse chase experiments, internal heterologous protein determination, electron microscopy and literature, a model was generated that could link the effects of ethanol cultivation to increased heterologous protein secretion. This model is graphically depicted in figure 2 and will be discussed in more detail below.

An integrated model of the effect of ethanol on cell state and secretion

Ethanol in the medium diffuses passively into the yeast cell where it is converted to acetaldehyde. This intracellular acetaldehyde can accumulate to high concentrations and is harmful to proteins inducing modifications and subsequently unfolded protein response (UPR) and general stress[22]. The acetaldehyde is converted to acetyl-CoA, which is further metabolised in the citric acid cycle (TCA).

The TCA is very strongly induced in ethanol cultivation[18,20], but compared to glucose it yields less carbon and energy. This and the strong metabolic and anabolic changes induce a strong change in redox-balance, induces reactive oxygen species and oxidative stress. It is not exactly clear what the structural impact on the mitochondria is of this up regulation of the TCA and oxidative stress.

The shortage of carbon and energy on ethanol and the removal of intermediates from the TCA for biosynthesis induce anaplerotic routes, to replenish it from other sources. Fatty acid oxidation (FA oxidation) in the peroxisomes is such a route that yields acetyl-CoA, which is transported to the mitochondria for the TCA metabolism. These activities were shown to be strongly up regulated[20]. Side products of this oxidation are aldehydes, which again induce

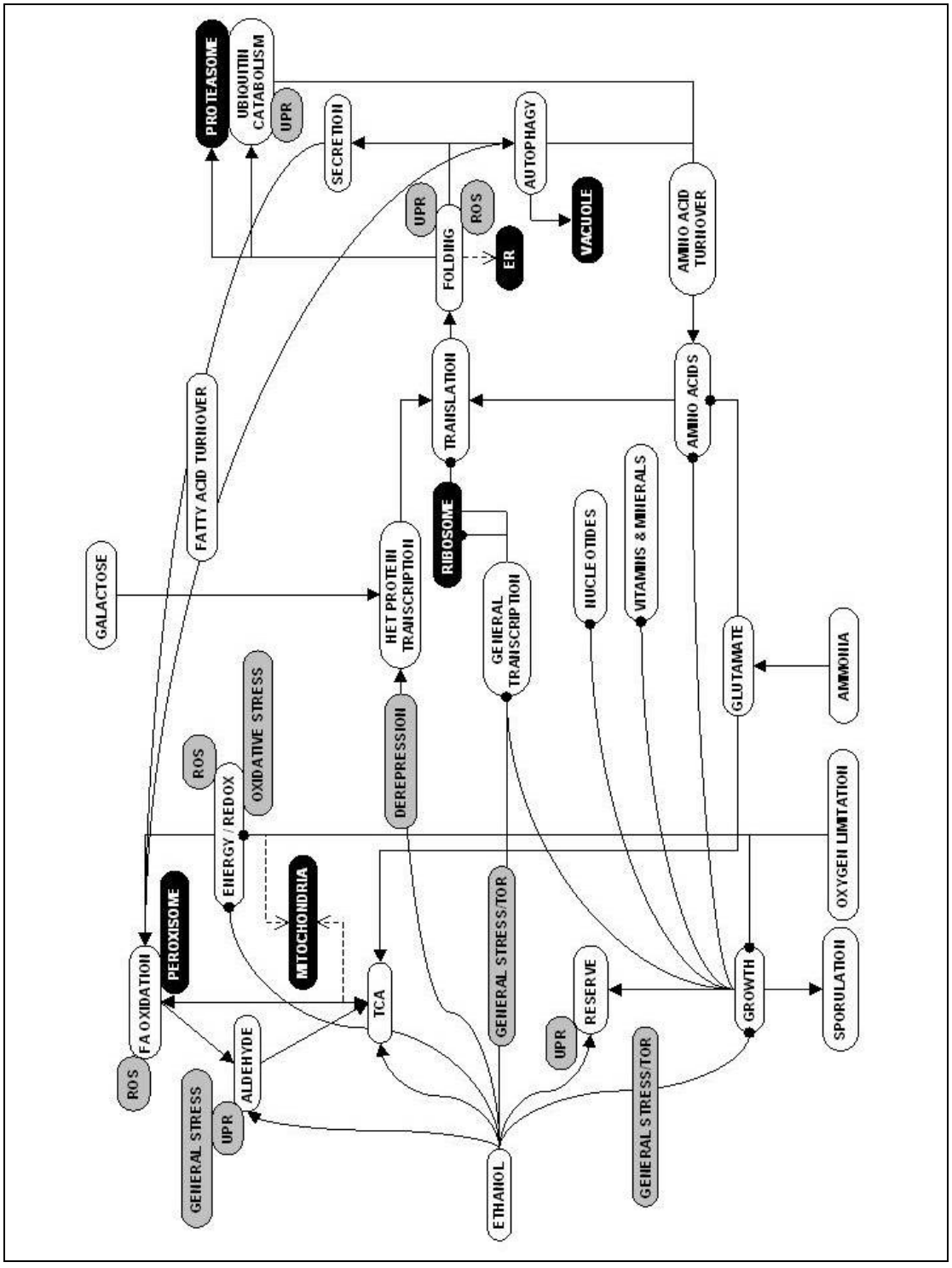

Figure 2.

An integrated model of ethanol induced increased heterologous protein secretion.

Black text boxes represent organelles. Grey boxes represent impulses and responses, either originating and/or effecting. Pointed arrows indicate a positive influence. Rounded arrows indicate a negative influence. Striped lines indicate to unknown effects.

On the left side are the metabolic and physiological aspects, on the right the protein production process related aspects.

UPR, and reactive oxygen species such as peroxide that induce oxidative stress and have a large impact on redox balance[23]. This also affects the folding processes in the ER as discussed below.
Ethanol cultivation also induces the reserve carbohydrate metabolism around trehalose and glycogen. Trehalose is induced upon unfolded protein response and has a chaperone function, protecting proteins against denaturing agents as well as stabilising membranes[24,25]. Furthermore, when the growth rate decreases it functions as reserve metabolite but can also be important in the cell cycle[26].

The general stress of ethanol cultivation and the lower carbon and energy yield decrease the growth rate[8]. As mentioned above, this induces the activity of the reserve metabolism. The ultimate effect of further decrease in growth rate is the induction of sporulation and meiotic responses[20]. At low growth rate, general transcription or endogenous and heterologous protein synthesis as well as ribosomal proteins are down regulated, which lowers the translational capacity. This down regulation is also witnessed in the metabolism of nucleotides. This down regulation has been ascribed to TOR-regulation[27]. TOR is the signalling pathway that is induced upon nutrient starvation, environmental changes and low growth rates[27,28]. Although we have shown that the reduction in growth rate itself cannot be the initiator of increased heterologous protein production.
When the growth rate decreases, as in the glucose decline phase, also the amino acid metabolism is down regulated. On ethanol however this down regulation is more affected through nitrogen catabolite repression due to the presence of ammonia which is metabolised to glutamate. This glutamate is subsequently used for anaplerotic routing to the TCA, an effect that has also been described in relation with TOR[28]. However in the decline phase of ethanol cultivation when the growth rate further decreases, the amino acid metabolism is up regulated[20]. This probably occurs, as a result of a shortage of amino acids for protein production and additional metabolic activity is necessary.

Galactose is transported from the medium into the cell where it is not subsequently metabolised, through the *GAL1* deletion, but serves as inducer for heterologous gene transcription. Ethanol seems to have a direct effect on this induction, as the amount of mRNA of the heterologous gene is higher on ethanol. This seems to be caused by the complete absence of glucose repression in ethanol cultivation and can contribute to a large extent to the increase on heterologous protein secretion[29].
The amount of heterologous transcripts has therefore increased on ethanol, while the amount of endogenous mRNA decreases because of the decreased growth rate. To process this increase of mRNA into large amounts of heterologous proteins sufficient translation capacity is required. Although on ethanol the ribosomal machinery is down regulated, there is apparently more than sufficient capacity to cope with this high flux[29].

The large amount of heterologous protein has to be folded correctly in the endoplasmatic reticulum (ER), which induces a strong UPR in both ethanol and glucose cultivation. Additionally the ethanol and aldehyde stress also induces an unfolded protein response, both cytosolic as in the ER, which can aid in the folding efficiency. More importantly, the redox state has a strong impact on protein folding, as disulphide bridge formation is highly dependent on this and the presence of ROS. The ethanol stress also induces a strong catabolic response as ER associated degradation (ERAD) is induced in the form of ubiquitin activity and induction of the proteasome. As mentioned before, because of lower growth rates and decreased protein synthesis, the amount of endogenous proteins is lower, while the total amount of unfolded proteins is probably lower due to UPR, REDOX and ERAD. These can increase the capacity and efficiency of heterologous proteins considerably.
Through these adaptations the flux of proteins to post ER transport increases. This is especially evident in the highly induced post Golgi transport. This transport is essential under stress as it directs harmful intermediates, unfolded proteins and unnecessary cellular components to the vacuole for catabolism. This is also witnessed in a strong autophagy up regulation on ethanol, which again, has been ascribed to TOR regulation[27,30]. The other branch of this highly induced trans Golgi network (TGN) is the secretion or exocytosis. As the whole TGN is strongly up regulated and the amount of endogenous proteins is lower, it adds strongly to the secretion flux of heterologous proteins, reflected in the strong increase of product in the fermentation broth[29].
The catabolic processes in the proteasome, autophagy and vacuolar degradation yield amino acids for the turnover of proteins for biosynthesis. The lipids of the transport vesicle membranes as well as other autophagised organelles are turned over by beta-oxidation in the peroxisomes, which also functions for the replenishment of the TCA.

The regulation of these processes is highly complex and cannot be dedicated to one or two transcriptional regulation activities. Many genes have multiple responsive elements in their promoter regions so that stress induction through Msn1/Msn2 stress responsive elements (STRE) regulated genes could well coincide with carbon source responsive elements (CSRE), YAP1 or others.
However, many of the witnessed effects like the regulation and redirection of the nitrogen metabolism[28], the decrease of translation and general protein synthesis machinery[27], autophagy[30] and even glucose derepression[28,31] are TOR regulated processes. Although TOR is a very broad description of regulation and contains many of the earlier mentioned regulatory pathways; it does seem to be the main effector. This implies that ethanol in fed-batch fermentation acts via a nutritional impact on the cell. But besides TOR, ROS and oxidative stress, UPR and glucose derepression seem to be also key factors inducing the ethanol effect on heterologous protein production.

To summarise this model of increased heterologous protein production in ethanol cultivation: the shortage of carbon and energy and the occurrence of damaging agents induce a very strong and broad stress response. The cells adapt by inducing transport and catabolic mechanisms and decreasing endogenous protein production. This increases protein turnover but also the flux of heterologous secretion. The resulting effect is an increased heterologous production under stressed conditions.

Aspects of DNA microarray analyses

Although the ethanol model is based to a large extent on DNA microarray data, the interpretation of this information in general is subject to great caution. Besides the experimental design, the variables and the reference[32], the data interpretation and the modelling of cellular responses should be performed carefully.

Although a gene is up regulated significantly in the data set this does not necessarily mean that this induction is a direct cause of increased cellular activity. In stead of activity, it could be that a lack of repression activates gene transcription. For instance, in ethanol cultivation a decreasing growth rate and the lack of monosaccharides induces hexose and galactose transporters. But the concentration of saccharides is extremely low and beside from some reserve metabolic activity there is hardly any sugar present in the cell[20].

Besides a lack of repressor it could also be that a gene is induced as a side effect of a different up regulation. The formate dehydrogenase genes are very strongly up regulated in ethanol cultivation although no formate is present and that the resulting protein is probably not active. But the effects of glucose deprivation and increased oxido reductase activity apparently co-induce these FHD genes [20].

A gene could also be not up regulated in the data set, but that does not necessarily mean that the effecting response is not present due to the transient response effect[16]. When a stress impulse is given, the cell responds through transcription factors. But after some time these factors and related genes are much less up regulated, although the cell activity has changed dramatically. For instance to adapt to the unfolded protein response, the endoplasmatic reticulum proliferation changes the organelle organisation. After this proliferation the response is less strong and only maintenance of the structure is necessary, which is less apparent in a DNA microarray if only a few time points are taken.

Finally, a gene can be up regulated significantly in the data set but this does not necessarily mean that the resulting protein response is the same[33]. Many of the effects are not regulated on a transcriptional level but by post transcriptional regulation such as post transcriptional modification, regulated protein catabolism, presence of sub units and co-enzymes, protein localisation and activation. Therefore proteomics as an additional tool to genomics would be very informative. Unfortunately however, these techniques and knowledge have not yet developed so far that a complete proteomic picture is available for *S. cerevisiae*[33].

DNA microarrays create an enormous amount of data and have yielded much information on the cellular mechanisms and are therefore very useful, but only if the data is analysed carefully, looking at groups of genes and general trends. More detailed gene regulatory analysis should be verified by other techniques, preferably on a proteomic level. Genomics is however an indispensable tool in cellular biology research and also for the optimisation of heterologous protein production.

Technical aspects of ethanol as carbon source

By changing from glucose to ethanol in a carbon source limited fed-batch fermentation, the product yield was increased significantly[7] (Figure 1A and B). Further studies on the effects of ethanol revealed that the accumulation of ethanol in the culture increased further this productivity[8] (Figure 1C). Although some results suggested that a higher ethanol concentration induced a higher specific production (dP/X), this was not actually shown in fed-batch cultures. It does lower the growth rate and thereby the specific productivity (dP/X/dt). Finally for the optimal process an accumulation level of approximately 1%v/v was chosen.
With accumulated ethanol in the broth the carbon source is no longer the growth limiting factor. Initially oxygen en medium components are available in excess, which implies that the cultivation is under maximal growth rate. This growth rate slowly decreases (figure 1C) as an effect of the ethanol inhibition, the culture is therefore not controlled by substrate or feed regime.
Only when the oxygen concentration becomes limiting in the decline phase the growth rate decreases and the culture is controlled by the oxygen transfer rate.

Under these unfavourable conditions of oxygen limitation, high cell densities, possibly poor availability of substrates, by-product accumulation and protease activity, the specific production remains maximum. The process can therefore be continued until the productivity decreases, which happens only under extreme conditions above 130 g/kg biomass[8]. Before that point, the maximum fermenter volume has normally been reached. For ethanol fermentations this creates several possibilities for continuing production by cell recycling or repeated fed-batch operation.

Repeated fed-batch

Single cycle fed-batch fermentations require a time consuming inoculum train, cleaning, sterilisation and down time of the fermentation vessel[34]. Therefore reuse of the productive biomass and the culture vessel could increase the overall productivity of a production process. This can be done in several ways, but the main principle is withdrawing volume from the bioreactor to continue the feeding and maintaining the high amount of biomass in a

productive environment[35] (Figure 3A). By changing the volume of withdrawal the efficiency of the process can be changed[36]. For instance, continuous withdrawing and feeding resembles a continuous culture under high cell density conditions (figure 3B) while large withdrawals generate more volume for downstream processing (figure 3C).

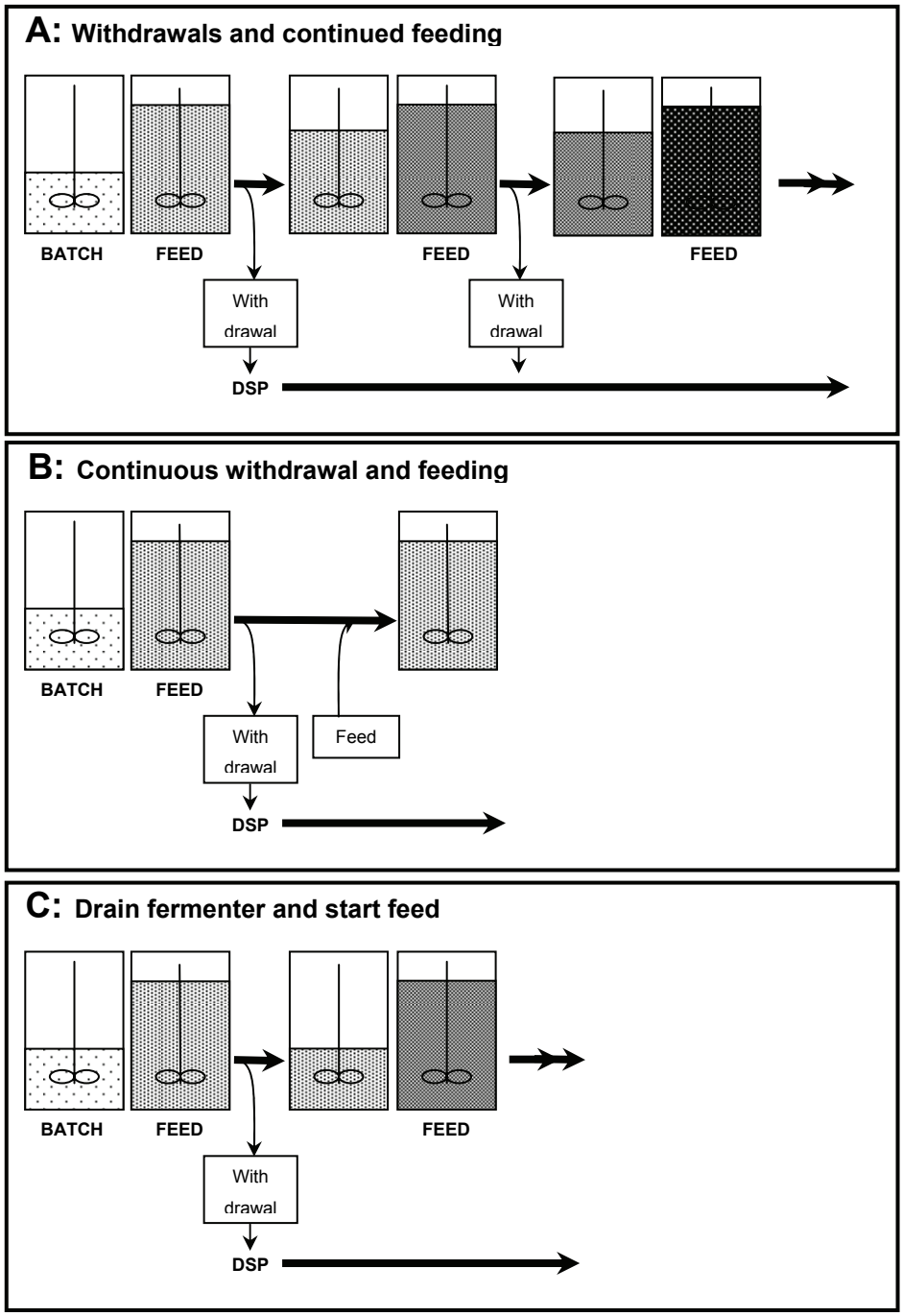

Figure 3. Part I

Various scenarios for repeated fed-batch fermentations. See text for explanation of different scenarios. Starting point is the step after the maximum fermenter volume has been reached. Withdrawing a volume for the vessel can be done in different sizes, having a different impact on the process. It is obvious from these scenarios that the logistics and the downstream processing capacity determine to a large extent the best repeated process.

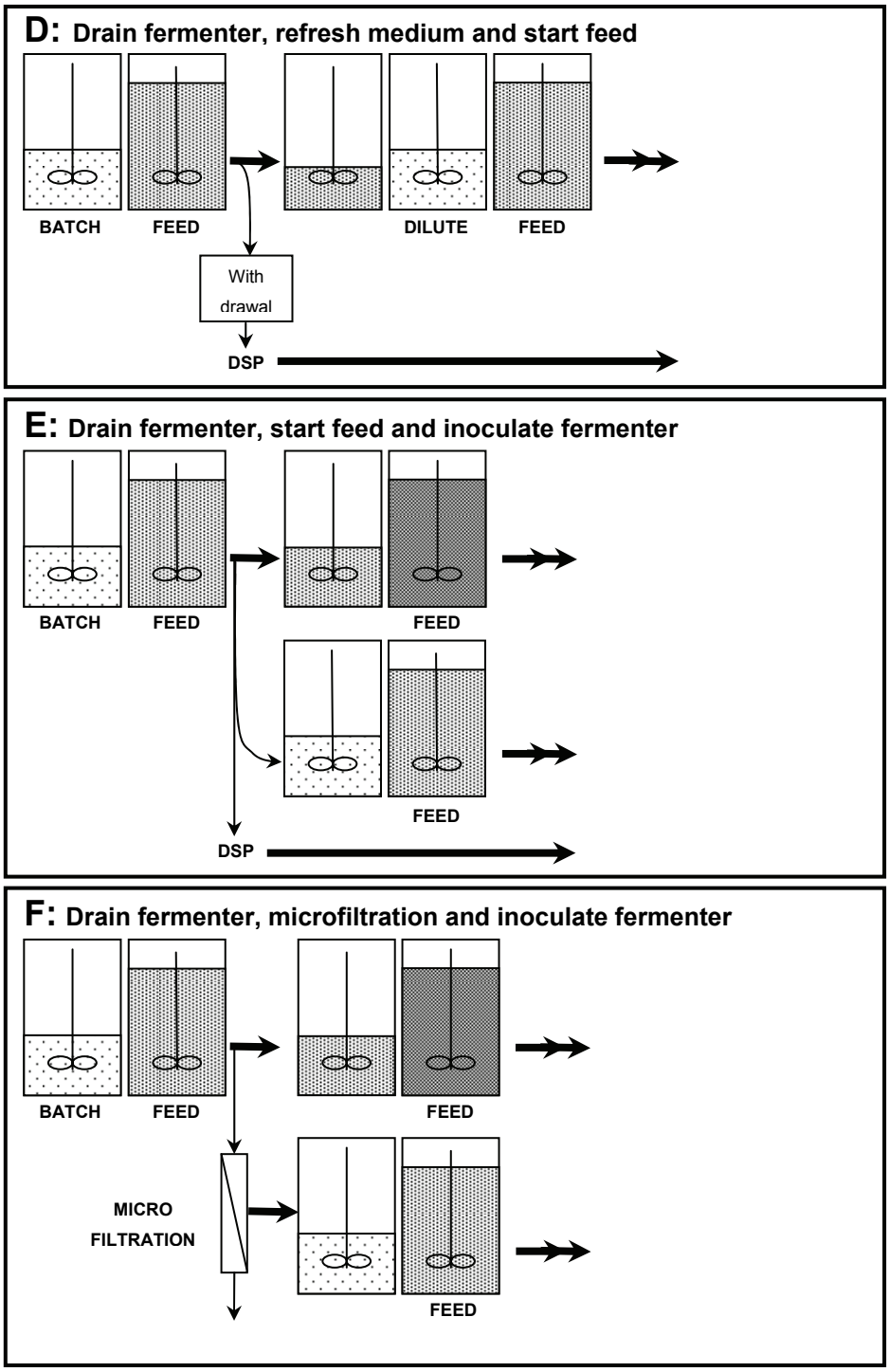

Figure 3. Part II

The remaining broth however keeps increasing in biomass and product concentrations but also inhibiting components like CO_2, metabolites and proteases[5]. Moreover the water activity decreases which can be detrimental to production[8]. Therefore refreshing the remaining medium could be very important to retain a high productivity (figure 3D). The withdrawn volumes can also be used for inoculating new fermenters at high biomass concentration which decreases the fermentation time (figure 3E), this is also possible by cell-retention

through microfiltration (figure 3F)[37]. The main issues for these processes are summarised in table II. Although extensive modelling of these cyclic processes is possible, most often the best operational method is found by trial and error[35] in accordance with downstream processing logistics.

Table II.
Issues regarding repeated fed-batch scenarios

Contributing factor	**Issues**
Active biomass retention	Large withdrawals decreases productivity
	Small withdrawals make better use of active biomass
Culture impact	High withdrawals increase culture shock
	Inoculation can induce Lag-phase
Logistics	Downstream processing minimal and maximal capacity
	Vessel capacity for withdrawals, refresh medium, high feed volume
	Production lines for inoculating second or more fermenters
Control	Temperature, oxygen and ethanol concentration
	Impact of withdrawal on cooling and aeration capacity
Inhibiting factors	Metabolites, toxic components, CO_2, product inhibition, water activity
Sterility and stability	More complex process induces infection risk
	Extended growth can effect strain and product stability

Up scaling

For a process to be economically achievable it is essential to scale it up as the volumetric production determines the cost and the price of the product. But productivity at larger scale is generally lower due to biological, chemical and physiological effects[4,38]. However, the most influential scale up effect can be traced back to transport phenomena due to increased mixing times in large scale vessels[39].

The productivity decrease is especially evident in high cell density fed-batch fermentations with glucose as sole limiting carbon source. There the feed is generally added at the top of the fermenter and due to larger mixing times the glucose concentration in that area of the fermenter is higher[40]. The aeration however is normally introduced in the bottom of the vessel where the oxygen concentration is significantly higher due to hydrostatic pressure, poor oxygen solubility and the increased mixing times[38,39]. This causes sub optimal mixing zones. At the top of the fermenter oxygen transfer has become limiting instead of the glucose concentration, also because of increased glucose consumption[40]. At the bottom of the vessel

the opposite occurs as glucose and other medium components are limiting or below the optimum while oxygen is in excess.

Cells pass through and reside in these zones depending on the mixing time. With *Saccharomyces cerevisiae* the high glucose concentration at the top results in ethanol formation and glucose repression on the *GAL7* promoter. Furthermore, oxygen depletion induces anaerobic growth and oxygen stress. Other zones around ammonia inlets and cool and heat areas can also induce stress responses[41]. Although the residence time may be brief, the response time for a metabolic change in *S. cerevisiae* is seconds[42] and an osmotic stress response in *E. coli* was witnessed within 15 seconds. To relax that same response a residence of 10 minutes in a more favourable zone may be neccesary[41].

Often though, no resulting effect is seen in the culture. The concentration of ethanol in the broth remains below detection either by consumption in the glucose starving zones or because the metabolic response is not complete due to a shorter residence time[40]. But the constant switching of environment can alter the physiological properties significantly. The change to glucose fermentation and the stress response mechanism lower the biomass yield, induces by-product formation and decreases the heterologous protein productivity[38,39,41].

Although these effects are strongly dependent on the vessel size and the tank configuration, to some extent they will always be apparent as long as the residence time is shorter than the induction time of a few seconds. Mendoza-Vega *et al.*[43] have described these effects to appear above 1000 L scale, others have reported a decrease in biomass yield of 7% by scaling up from 10 L to 120 m^3 [41]. Bylund *et al.*[40] even have shown a variation of 5% lower biomass yield between different zones within one large scale vessel although this was performed with an *Escherichia coli* culture.

The ethanol fed-batch fermentation process has several features that make it much more suitable for up scaling. In contrast to glucose growth, there is no overflow metabolism on ethanol. And also glucose repression of the *GAL7* promoter is not possible. The biomass yield and the induction of heterologous protein expression will therefore not change.

The ethanol concentrations will be higher around the feed inlet than in the rest of the vessel due to the pulsed feed and the higher mixing time. But it has been reported that the increase of ethanol concentration has a positive effect on specific production. Also, the presence of oxygen depleted zones has no effect on production and biomass yield, although the growth rate is decreased under these circumstances[8]. Finally, there will be no on and off switching of stress responses and consequent adaptations as the cell is in a constant state of stress due to the ethanol effect.

In ethanol cultivations, the biomass yield remains the same for all scales, although fermentation time could increase because of the lower growth rates. The production yield however could even increase in scaling up because of the ethanol effect. These features of glucose and ethanol fermentations have indeed been witnessed in several scaled up processes (data not shown).

For the application on large scale, ethanol does have some drawbacks as it is subject to taxation and per Cmol it is more expensive than glucose or molasses. In addition, ethanol cultivation can only be performed in explosion proof facilities. With the increased use of methanol induced host strains such as *Pichia pastoris* this is becoming less of an issue. Furthermore, the yield increase over glucose can be so dramatic that it can be a cost effective production alternative.

Further improvements on heterologous protein secretion

The research and development of an ethanol production system for heterologous proteins has yielded several points for further improvement.

Although the ethanol effect increases the secretion efficiency, still much internal protein was shown to be present. This implies that the production could still be further improved by changing the production system, from expression cassette, promoter system, signal sequences to improved amino acid sequences of the heterologous proteins self.

It would be interesting to see what the relationship is between ethanol and these different optimisations. Also the relationship between stress and nuclear processes such as transcription and RNA processing is still a much unknown area.

In general it could be possible that the sub-stresses of the ethanol effect can be further fine tuned to improve the ones that are more essential for heterologous protein production, for instance by chemical intervention or by genetic modification.

For the cultivation process itself it was shown that medium optimisation could further enhance production. The addition of fatty acids or metabolic intermediates could improve the carbon and energetic yield. But also the balancing of nitrogen and amino acids in the several phases of cultivation could be beneficial, just as the fine tuning of vitamins and trace metals.

However, any improvement of the carbon and energy metabolism might have the opposite effect on the heterologous protein productivity, as it is strongly dependent on the stress state of the cell.

Therefore ethanol cultivation seems to be the most optimal mode of operation as it is a very robust carbon, energy and stress source, all in one.

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Samenvatting

De productie van bakkersgist (*Saccharomyces cerevisiae*) voor het rijzen van deeg is een van de oudste fermentatie processen in de geschiedenis. Het wordt gekarakteriseerd door de productie van ethanol en kooldioxide door de fermentatie van suikers.
Tegenwoordig wordt *S. cerevisiae* ook gebruikt voor de productie van recombinante eiwitten die van groot belang zijn voor industriële toepassingen zoals de farmaceutische markt en industriële enzymen. Deze zogenaamde heterologe eiwitten worden normaliter geproduceerd in fed-batch fermentaties met hoge celconcentraties, waarin glucose als enige koolstofbron wordt gebruikt. Echter, een overmaat van deze suikers induceert de productie van ethanol. Deze ethanol fermentatie is recentelijk geanalyseerd met behulp van DNA microarrays, waaruit bleek dat de regulatie van een opmerkelijk groot aantal genen zowel sterk was verhoogd als verlaagd. De meeste van deze veranderingen worden beschouwd als ongewenst en betiteld als stress respons en in de analyses bleken dan ook veel stress eiwitten te zijn geïnduceerd en de opbrengst van biomassa op de koolstofbron te zijn verlaagd.

Om het productieproces van heterologe eiwitten te verbeteren is het gehele productiesysteem aan een grondige analyse onderworpen. Het bleek dat juist de cultivatie op ethanol in een simpel batch fermentatieproces een gunstig effect had op de opbrengst van heterologe eiwitten door *S. cerevisiae*. Dit geldt in het bijzonder voor de eindfase van het proces en is aangetoond voor verschillende recombinante eiwitten. Vervolgens is dit proces verder ontwikkeld om de eigenschappen van ethanol cultivatie ten volste te benutten. Daarvoor zijn eerst deze karakteristieken bepaald in batch en fed-batch fermentatie experimenten door de invloed op de productiviteit te onderzoeken van de groeisnelheid, de zuurstof concentratie en de ophoping van ethanol in de cultuur. Dit resulteerde in een verbeterd productie protocol.

Vervolgens hebben we onderzocht hoe *S. cerevisiae* in staat is om de heterologe eiwit opbrengst te verhogen terwijl het groeit onder stress omstandigheden en onder een suboptimaal gebruik van de energie. Dit is gedaan door allereerst te focussen op de metabole en fysiologische staat van de gistcel en vervolgens door het bestuderen van de eiwitproductie en secretieprocessen in de cel. Hiertoe zijn verschillende methoden gebruikt: DNA microarrays om een totaal beeld van de veranderingen te krijgen, de constructie van een mutant van het heterologe eiwit (variabel domein van Cameloide antilichamen, VHH's) en elektronen microscopie (EM).

De DNA microarrays bevestigden dat vele processen in de cel dramatisch veranderen. Het stikstof- en vetzuurmetabolisme bleken een belangrijke rol te spelen door intermediairen naar het centrale metabolisme te dirigeren om het tekort aan koolstof en energie uit ethanol te

compenseren. Ook is een verhoogde expressie gevonden van genen die gerelateerd zijn aan eiwitkatabolisme. Daarentegen was de ERAD respons niet geïnduceerd, hoewel dat normaliter wel gebeurt in heteroloog eiwit producerende *S. cerevisiae*.
Ook is gevonden dat ondanks het feit dat de glucoseconcentratie in het fed-batch proces nagenoeg nul is, de *GAL7* promotor voor het gen dat voor het heterologe eiwit codeert zorgt voor een veel hogere transcriptie. Verder is een sterk effect aangetoond van de redox-staat en de oxidatieve stress. Mutanten van VHH's toonden aan dat juist VHH's met vouwingsproblemen door de mogelijke vorming van ongewenste zwavelbruggen, aanzienlijk beter worden geproduceerd met ethanol als enige koolstofbron in plaats van glucose. Echter, de productieverhoging is niet beperkt tot dit fenomeen. Ten slotte heeft EM een aantal opmerkelijke aspecten laten zien, de belangrijkste: de hoge aanwezigheid van heteroloog eiwit en het endoplasmatisch reticulum eiwit BiP in een vergrote celwand.

Enerzijds hebben deze observaties geresulteerd in een compleet nieuw proces voor de productie van heterologe eiwitten, dat toegepast is op grote schaal (>10.000 L). Anderzijds is het mogelijk gebleken om op basis van DNA microarrays, mutantenonderzoek en EM observaties een kwalitatief model op te stellen om de verhoogde eiwit opbrengst op ethanol te verklaren.

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Ach, een beetje sentiment kan geen kwaad.
Een kip is immers ook geen mus.
Dus.

Curriculum Vitae

De auteur van dit proefschrift werd geboren op 19 december 1971 te Boxmeer. Na het behalen van het V.W.O. diploma aan het Elzendaalcollege te Boxmeer in 1990, werd in datzelfde jaar begonnen met de studie Bioprocestechnologie aan de Landbouw Universiteit Wageningen. Naast een stageperiode bij de University of Houston in de Verenigde Staten, waar hij gewerkt heeft aan de productie van antilichaam fragmenten in *E.coli* en aan het screenen van chromatografie matrices, heeft hij twee afstudeervakken afgerond. Het eerste bij de vakgroep Industriële Microbiologie (Dr. Jasper Kieboom en Prof. Dr. Ir. Jan de Bont) waar de microbiële productie van catecholen door *Alcaligenes sp.JB1* in continu culturen is bestudeerd en een tweede bij AVEBE in Groningen vanuit de vakgroep Proceskunde (Dr. Leon Marchal en Prof. Dr. Ir. Johannes Tramper) waar de invloed van amylolytische afbraak van zetmeel op het saccharidenspectrum is onderzocht. In 1997 heeft hij zijn ingenieurstitel behaald.

In 1998 is hij werkzaam geweest bij Introgene te Leiden als Chromatography Engineer en vervolgens is hij in 1999 aangesteld bij Unilever Research and Development in Vlaardingen, als Fermentation Engineer bij de afdeling Biotechnology Application Centre (BAC). Vanaf 2001 heeft hij deze werkzaamheden gecombineerd met de uitvoering van het promotie onderzoek dat in dit proefschrift beschreven is, in samenwerking met de vakgroep Cellular Architecture and Dynamics van de Universiteit Utrecht (Prof. Dr. Ir. Theo Verrips). In 2003 is hij binnen BAC BV als Manager Process Development in Naarden aangesteld. Vanaf juli 2006 is hij werkzaam als Scientist bij de afdeling Small Scale Cell Culture and Development bij Diosynth te Oss, onderdeel van Organon NV.