

Heterologous expression and purification of wheat storage proteins in the yeast *Saccharomyces cerevisiae*

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Abbreviations

% (w/w)	percent weight per weight	NZ	NZ-amines (protein hydrolyzate)
μ	specific growth factor	OD	optical density
2xDS	two-fold concentrated DS-medium	OUR	oxygen uptake rate
ATPS	aqueous two phase system	PBS	phosphate buffer saline
BSA	bovine serum albumine	PD	phase diagram
CA	Casamino Acids (protein hydrolyzate)	PEG	polyethylene glycol
DAPI	4,6-diamidino-2-phenylindole dihydrochlorid	PMSF	phenylmethylsulfonyl fluoride
DCW	dry cell weight	rDNA	recombinant desoxyribonucleic acid
dH ₂ O	deionized water	RNA	ribonucleic acid
DNA	desoxyribonucleic acid	rpm	revolutions per minute
DTT	dithiothreitol	RT	room temperature
EDTA	ethylenediamintetraacetate	SDS- PAGE	sodium dodecyl sulfate polyacrylamid gel electrophoresis
ER	endoplasmatic reticulum	TLC	thin layer chromatography
gDCW l ⁻¹ h ⁻¹	biomass productivity	sok	suppressor of killing
GTP	guanosine triphosphate	vvm [l l ⁻¹ min ⁻¹]	volume air per reactor volume and minute
H	hourly increase factor	WSP	wheat storage protein
HCD	high cell density	Y _{x/s}	specific biomass to substrate coefficient
HCDF	high cell density fermentation	π	productivity- gram product per hour and liter
HMW	high molecular weight subunit of wheat storage proteins		
hok	host killing		
K	partition coefficient		
LMW	low molecular weight subunit of wheat storage proteins		
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)		

Standard SI units are used throughout.

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I. THEORETICAL PART

Production of recombinant protein by high cell density fermentation (HCDF)

A comparative view of prokaryotes (*E. coli*) and eukaryotes (*S. cerevisiae*)

1 Introduction

Over the last centuries, biotechnology focused on the production of a variety of biological compounds. At the beginning the major aim was the production of food and products of the primary metabolism, e.g. ethanol, citric or lactic acid. Secondary metabolites (e.g. antibiotics) became important several years later (after 1940). Today the degradation or conversions of biological active compounds, needed for waste water treatments or for the production of complex compounds are of interest. Before the use of recombinant DNA technologies to control cellular metabolism and to create new compounds, cellular activities were increased by mutagenesis and subsequent selection. With the extension of knowledge of genomic data and the development of suitable genetic tools, the production of recombinant proteins mainly for diagnostic or medical purposes became an increasingly fast growing sector.

In order to achieve high yield of recombinant proteins two major approaches are feasible. The first is the improved productivity by process design for selected production strains. The second, but not less important approach is a genetic improvement of a host vector system that allows higher product formation, without intervening fast cell growth.

The increase of cell density represents a simple approach for improving the productivity several times. The highest biomass yield can be achieved with the so called “high cell density fermentation” (HCDF). The advantage of HCDF in contrast to classical cultivation is simple: HCDF gives the opportunity to produce microbial products in a much smaller volume. This has great process related and financial consequences. Smaller reactor volumes result in a decreased energy input (heating, sterilization, stirring, pumping and piping) and resource throughput (water, waste water). The equipment for downstream processing (separator, centrifuges, disintegration, chromatography and filtration devices) can be scaled down or may become unnecessary. However, these advantages are accompanied by several problems. Solubility, instability and volatility of substrates and products are the main problems. Moreover, substrate limitations or inhibition of growth by substrates or products represents further hurdles. In addition, the production of CO₂ and heat, the high oxygen demand and increasing viscosity play important roles. High production of growth inhibitory byproducts as

acetate (*E. coli*), propionate (*B. subtilis*), lactate (*L. lactis*) or ethanol (*S. cerevisiae*) has to be diminished or technological efforts are necessary to remove them during cultivation.

Nowadays major hosts for the expression of foreign proteins are *Escherichia coli*, *Bacillus spec.*, filamentous fungi, yeasts, insect cells and mammalian cells. The choice of a suitable host depends on cell growth characteristics, expression levels, product localization, posttranslational modifications and biological activity of the particular product. The selection of the most suitable expression system requires several decisions in terms of process design, reactor design and other economic considerations. Factors that affect product recovery (e.g. localization, yield and properties of the product) are of highest importance, because recovery steps represent the major part of total process costs.

E. coli and *S. cerevisiae* became genetic model organisms and important hosts for recombinant protein expression, because their cultivation is simple and their genetic background is well characterized. These model organisms, which represents a prokaryotic and an eukaryotic will be presented and compared for the production of recombinant proteins in HCDF in the following sections.

2 Comparison of *E. coli* and *S. cerevisiae*

E. coli can produce high yields of recombinant proteins. Its cultivation is simple and growth is very fast with a specific growth rate higher than 0.35 h^{-1} . *S. cerevisiae* exhibits lower protein yields and slower growth rates than *E. coli*. Nevertheless, it is also able to grow on simple media to high cell density and cultivation complexity is only slightly different to *E. coli*.

While complex proteins are correctly folded by *S. cerevisiae*, the inability of *E. coli* to do so often leads to aggregates (i.e. inclusion bodies) or to degradation of the recombinant protein. Moreover, the lack of post-translational modifications (e.g. glycosylation), an effective secretion system and the absence of disulfide-bond formation are other main disadvantages of *E. coli*. The necessity of extensive testing for endotoxins and cell wall pyrogens which has to be performed if products should be clinically used (Mendoza-Vega et al., 1994) is another drawback of *E. coli*. *S. cerevisiae* is clearly favored, if folding of complex proteins and posttranslational modifications are needed. Many genetic tools and expression systems have been developed for both hosts to achieve high protein expression. Although *S. cerevisiae* is in comparison to others (e.g. *bacillus*) not a good secreter, hosts were engineered to secrete recombinant proteins. The low amount of only 0.5% of host related proteins secreted by yeast (Vasavada, 1995) allows an simplified purification, because of low host-cell protein contamination. Although most yeasts are able to perform post-translational modifications such as protein folding, glycosylation, disulfide-bond formation, removal of the N-terminal

methionine, acetylation, myristylation, farnesylation and proteolytic processing, the glycosylation pattern significantly differs to those of higher eukaryotes. Yeasts often tend to hypermannosylation, which does not fit to the original structure and thus different immunological consequences could be presumed in human. Nevertheless, yeasts combine the uncomplicated cultivation of prokaryotes with the post-translational modifications similar to higher eukaryotes. Approaches for retrieving a human like glycosylation pattern are the “humanization” of the host organism (Choi et al., 2003; Wildt and Gerngross, 2005).

The fact that yeasts cannot be contaminated with viruses or retroviruses and that the organism is generally regarded as safe (GRAS) are further advantages of the yeast host system and emphasize it in contrast to bacterial or mammalian cells.

If post-translational modifications are not required, *E. coli* remains the most attractive system for recombinant protein production at industrial scale, in spite of the mentioned disadvantages. This is because many eukaryotic proteins are biologically active even if they are not modified. Moreover, some efforts were made to overcome certain major disadvantages of *E. coli* as a host strain, e.g. disulfide bond formation by reductase deletion (Bessette et al., 1999). Although high yields of recombinant proteins are achievable with *E. coli*, inclusion body solubilization and protein folding represent major cost factors for downstream processing.

3 Host-vector system suitable for HCDF

The successful expression of recombinant proteins depends on the vector systems. Detailed information for the configuration of host vector systems were given by Makrides (1996). The choice of a suitable strain is firstly focused on process related properties, as growth characteristics within selected media as well as the secretion of recombinant protein. Biosafety, information availability and economic consideration are further important factors. Laboratory strains often carry genetic modifications, which are of interest for genetic studies. However, these modifications can cause several drawbacks for an industrial use like defects in growth and protein biosynthesis. These strains are often the result of a mutagenesis with subsequent selection of particular properties (e.g. secretion or deficient proteolysis) (Hashimoto et al., 2005). Strain construction and maintenance may result in mutations or strain crossing events that lead to deficiency in respiration and proteolysis and sensitivities to temperature and osmolarity.

Before a strain is chosen for the production, a full strain characterization is necessary. Nutritional requirements, protease activity, auxotrophic marker, kinetic parameter (μ , $Y_{X/S}$) and sensitivities to culture conditions should be investigated. After transformation, further

characterization is necessary, because different clones from one transformation can show different growth behavior and recombinant protein expression.

In order to understand recombinant protein production in the different hosts, simplified basic vector structures are explained below. Prokaryotic and eukaryotic vectors have some similar features, but there are also important differences as presented in Figure 1 and Figure 2.

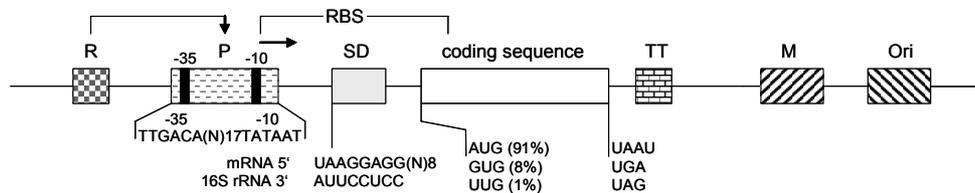


Figure 1: Basic structure of prokaryotic expression vector.

The sequence of a prokaryotic expression vector consists of regulatory sequence (R) that can be located on a plasmid or integrated into the host genome, a promoter sequence (P) and the Shine-Dalgarno-Sequence (SD) that interacts with the ribosome and represent the ribosome binding site (RBS), the coding sequence of the gene of interest with start and stop codons, the terminator (TT) for efficient stop of transcription and thus transcript stabilization, the selection marker (M) for selection of plasmid containing cells, and the origin of replication (Ori) for the amplification of the vector. Figure adapted (Makrides, 1996).

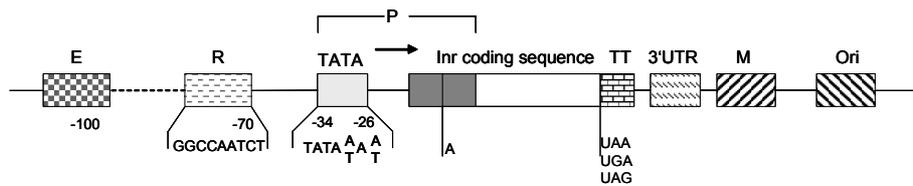


Figure 2: Basic structure of eukaryotic expression vector.

The eukaryotic vector consists of a possible enhancer (E) that enhances transcription, a regulatory sequence often found 70bp upstream, a promoter sequence containing the TATA-Box also upstream of the start codon and a Inr-element starting upstream and containing the start codon of the coding sequence, a terminator (TT) for efficient translation termination, a 3' untranslated region that determines also the transcript stability, a selection marker (M) and an origin of replication (Ori) if not chromosomal integrated.

Metabolic burden of replication, plasmid maintenance and product formation are important factors for vector construction. It has been shown that the metabolic burden is disproportional large with respect to the amount of heterologous protein produced (Gorgens et al., 2001). It increases with the expression of foreign genes and, more importantly with competition for transcription or translation factors and biosynthetic precursors. Even the production and processing of a recombinant protein itself bears a stress inducible risk (Hoffmann and Rinas, 2004), because of its interaction with host cell proteins. Vector construction should heed these factors to avoid growth limitation. The improvement of the genetic background to overcome limitations in biosynthetic precursors and energy requirement plays an important role to recover growth limitations due to metabolic burden (Flores et al., 2004).

Many different parameters have to be considered for successful vector design. Plasmid copy number (del Solar and Espinosa, 2000), transcription termination (Nudler and Gottesman,

2002), mRNA-stability (Kushner, 2004), translation initiation (Fuchs, 1999), enhancement and termination (Mugnier and Tuite, 1999), as well as codon usage (Saier, 1995) are important for efficient heterologous protein production. In addition, the final intra- or extracellular localization of the product has immense influence on process design. Long cultivation time and high concentration of an accumulated product could lead to interactions with host cell components, if the product is intracellularly expressed. Therefore, secretion of the product is commonly favored and efforts have been made to efficiently secrete recombinant protein from *E. coli* as well as *S. cerevisiae*.

Because of the complexity of all these parameters, the following sections will only focus on plasmid maintenance and transcription promoters, which are the two most important aspects for HCDF. Plasmid maintenance is very important for retaining high heterologous protein yields even after long time cultivation and on the other hand, transcription promoters have huge influence on process design and are decisive for a certain strategy.

3.1 Plasmid maintenance

Beside segregational plasmid stability, structural plasmid stability is also very important. However, structural plasmid stability, which comprises correct replication and unchanged nucleic acid sequences will not be considered in the following sections and can be found elsewhere (Friehs, 2004).

The importance of plasmid maintenance and segregational stability is given by the fact that a loss of only 1% per generation will result in 8.4% reduced recombinant product after 17 generations (Hensing et al., 1995). In the absence of any selective pressure, gene expression and plasmid maintenance are influenced by the length of the cloned DNA fragment, the growth temperature, and the plasmid copy number (Zielenkiewicz and Ceglowski, 2001), but not by the plasmid size. However, the toxicity of the recombinant protein, determined by structural features, often exceeds and thereby masks the influence of metabolic burden on maintenance. Thus different proteins will give different plasmid maintenance behavior (Corchero and Villaverde, 1998).

Beside its huge influence on gene dosage, the plasmid copy number also affects plasmid maintenance. If the distribution of plasmids is random, the probability that one daughter cell is plasmid-free is given by the equation: $P_0=2^{(1-n)}$ (Nordstrom and Austin, 1989) with n representing the plasmid copy number at division. In the case of low-copy number plasmid with $n=4$, there is a probability that one out of eight daughter cells is plasmid free, whereas if $n=20$, only two out of 1 million cells are plasmid free. *S. cerevisiae* multiplication is based on non-uniform budding. This leads to uneven distribution of the plasmids. Therefore,

segregational instability leads to higher probability of losing the plasmid (Gupta et al., 2001) compared to *E. coli*.

Metabolic burden is a major reason for the loss of segregational plasmid stability, i.e. cells without plasmids overgrow plasmid-harboring cells. Interestingly, a metabolic burden can be used to increase segregational plasmid stability: chromosomal pili synthesis controlled by the *tac*-promoter and repressed by the plasmid related protein *lacI* in *E. coli* leads to an increased metabolic burden if the plasmid and thus the repressor becomes lost (Kimberly L. Ogden, 1992).

3.1.1 Toxic compound resistance

Resistance to toxic compounds is commonly used for the selection and maintenance of plasmids. Ampicillin or kanamycin resistance (Philippon et al., 2002) are the most important antibiotic resistances used in prokaryotic hosts such as *E. coli*. The G418 resistance (Hadfield et al., 1990) is often used on shuttle vectors, since the gene-product is active in eukaryotic model organism *S. cerevisiae* as well as in prokaryotes. The constitutive expression of a resistance gene represents a metabolic burden which, if removed or decreased in expression, significantly increased the yield of recombinant protein (Cranenburgh et al., 2001).

High concentrations combined with a short half-life and high prices as well as hindered downstream processing and high risk of spreading to the environment (horizontal gene transfer) (Wadman, 1996) are further disadvantages of toxic compound resistances. Although high segregational plasmid stability can be achieved by this approach, resistance to toxic compounds is not suitable for HCDF.

Another kind of resistance is provided by the expression of genes mediating a tolerance against medium compounds, which are present within the cultivation medium at high concentrations. CUP1, the gene for metallothionein, used in copper sensitive *S. cerevisiae* strains is one example for this mechanism (Jeyaprakash et al., 1991). However, the disadvantages of a high metabolic burden and addition of substances to the medium are similar to antibiotic resistance marker. A number of toxic heavy metal resistance systems are known for bacteria (Silver and Ji, 1994), but environmental consequences for waste water prohibits the usage. Hyperresistance against formaldehyde is mediated by the gene *SFAI* (mitochondrial long chain alcohol dehydrogenase) in different yeast strains (Schmidt et al., 1997). Despite its unhealthy properties, formaldehyde provides the advantage of its biodegradation to CO₂ and hence reduces downstream costs compared to antibiotics. Nevertheless, its suitability for HCDF is questionable.

3.1.2 Complementation of chromosomal mutation

A number of alternatives for the use of resistance genes to select for and stabilize plasmids in fermentations have been described by [Hanak and Cranenburgh \(2001\)](#). The most important is the complementation of chromosomal mutations of essential genes. This strategy is efficiently used in both hosts *E. coli* and *S. cerevisiae*. Two main approaches having different process relevant properties were used. The first approach uses mutations, which can be complemented by supplements added to the cultivation medium. In this system, an auxotrophic marker encodes intermediates at low levels (e.g. amino acids or growth factors), which are used for plasmid maintenance. In that way the genes for the biosynthesis of tryptophan ([Matsui et al., 1990](#)) or threonine ([Nudel et al., 1989](#)) complement commonly deletions in *E. coli* and *S. cerevisiae* ([Pronk, 2002](#)). Auxotrophic markers not only substitute antibiotic resistance genes as selection markers, but could also improve segregational plasmid stability and heterologous protein expression in *E. coli* ([Fiedler and Skerra, 2001](#)). A way to increase plasmid copy number and thus the recombinant protein expression is the partial promoter deletion of complementing genes (e.g. Ura3d, Leu2d), which was shown for *S. cerevisiae* ([Patel et al., 2003](#)). These deletions resulted in decreased expression and increased selection pressure to multiple plasmids. However, the usage of auxotrophic markers for industrial, large scale production is not advantageous, because lysis and secretion of selection compound leads to high number of plasmid-free cells, e.g. tryptophan ([Schneider et al., 2005](#)). In addition, complex media cannot be used. A two step cultivation ([Gupta et al., 2001](#)) where cells are cultivated in selective media first and transferred continuously to the second, amino acid limited feeding stage is an approach to increase the usability of auxotrophic markers for HCDF. The second approach (non-auxotrophic marker) uses chromosomal mutations that cannot be complemented by a substrate. For example genes encoding essential steps in different synthetic pathways, e.g. pyrF (orotidine-5'-phosphate decarboxylase) in bacteria ([Schneider et al., 2005](#)), essential in pyrimidine biosynthetic pathway, or infA (coding for translation initiation factor 1, IF1) in *E. coli*. This approach gives highly stable plasmids also during long time cultivation ([Hagg et al., 2004](#)). A similar method is used in *S. cerevisiae* by the complementation of fructose-1,6-bisphosphate aldolase (FBA1) ([Compagno et al., 1993](#)). Loss of plasmids immediately leads to a termination of growth. The possibility of using a complex medium, what is favored in industrial productions makes these last described approaches highly suitable for HCDF.

3.1.3 Plasmid addiction systems

Plasmid addiction systems denote another type of plasmid maintenance strategy. Maintenance is achieved, because a plasmid loss causes cell death. The post-segregational killing mechanism (PKS) is one important representative of this maintenance type. This approach is derived from naturally-occurring plasmids of *E. coli* and consists of a toxin and of an associated antitoxin. The loss of the plasmid kills plasmid-free cells due to the longer half life of the toxin molecules (Cooper and Heinemann, 2000; Brendler et al., 2004). While acting on an earlier level of protein synthesis and therefore reducing the metabolic burden, the hok/sok-system is a similar plasmid maintenance strategy. Stable hok mRNA is inhibited by unstable sok antisense RNA in *E. coli* (Thisted et al., 1995).

The plasmid carrying restriction modification system is another example for a plasmid addiction system. A plasmid encoded methyltransferase prevents restriction of host DNA by chromosomal restriction endonuclease (Kulakauskas et al., 1995). This system has been described for *E. coli* and *Bacillus subtilis*, but it can be applied in other hosts. The usage of these plasmid addiction systems in HCDF was not investigated so far. However, high suitability can be presumed, because of unchanged metabolic burden.

The metabolic burden of commonly used plasmid maintenance strategies in *E. coli* can be reduced by repressor titration. Plasmid maintenance is achieved by using the lac operator sequence as selectable marker (Cranenburgh et al., 2004). The applicability has been demonstrated in HCDF (Durany et al., 2005). Dry cell weights of approx. 50 g l⁻¹ and an accumulation of approx. 30% of recombinant fuculose-1-phosphate aldolase of total intracellular protein could be achieved without plasmid loss.

Another system that represents a plasmid addiction, but only at certain cultivation condition is the provision of enzymes for the metabolization of strain unsuitable substrates e.g. sucrose. The *SUC2* gene (invertase) from *S. cerevisiae* enables the yeast *Y. lipolytica* to consume sucrose as single carbon source (Nicaud et al., 1989). This is an easy way with low metabolic burden and high maintenance rates. The use of non-secreted proteins increases the suitability for HCDF, because plasmid-free cells benefit from secretion. Several different strategies for “plasmid addiction systems” were reviewed by Zielenkiewicz and Ceglowski (2001).

In summary, plasmid addiction systems represent highly suitable strategies for plasmid maintenance in HCDF, especially if metabolic burden is low. Moreover, the strong selection pressure provides high segregational plasmid stability.

3.1.4 Active partitioning

Active partitioning is found for plasmids in both, *E. coli* and *S. cerevisiae*. High segregational plasmid stability is obtained without selection pressure and even at long cultivation times. Active partitioning systems were not used for large-scale production so far, because these plasmids are low copy number plasmids. New approaches to achieve active segregation were found within the last years. One mechanism is known for the bovine papilloma virus type 1, where the viral E2 protein provides segregation of the viral genome to the host daughter cells through binding to chromosomes; especially to a bromodomain protein (Brd4). This system has been applied to the yeast *S. cerevisiae* and contributes to a stable plasmid maintenance without selection pressure (Brannon et al., 2005).

Similar systems are known for *E. coli* plasmids, e.g. bacteriophage P1 (Li and Austin, 2002) or F-plasmid. However, suitability for HCDF is limited, because of low protein production resulting from low plasmid copy numbers. The high number of proteins which are necessary for stable plasmid maintenance represents a high metabolic burden, thereby reducing the yield and biomass production.

3.1.5 Conclusion for plasmid maintenance

During long time fermentation, even stable plasmid constructs can be lost. The reasons for this phenomenon are diverse. Toxic products may accumulate or the metabolic burden might be too high. Moreover, non-selective or semi-selective conditions as well as segregation failure during cell division or recombination events between chromosomal and plasmid based sequences might reduce plasmid stability. Furthermore, the cultivation conditions are important. Both factors can have a significant effect on plasmid maintenance. Limitation in carbon, nitrogen and phosphate may result in decreased segregational plasmid stability as has been shown for *S. cerevisiae* by Mendoza-Vega et al. (1994).

Plasmid maintenance strategies that combine high maintenance rate and low metabolic burden will result in highest product yield in HCDF. These features have so far been realized by non-auxotrophic marker and several plasmid addiction systems.

Beside the above discussed plasmid maintenance systems also the expression level and the time point of expression have a wide influence on protein output. Therefore, suitable promoter design for the use in HCDF is discussed in the following section.

3.2 Promoter

Promoters play a decisive role for the process design, especially in HCDF. High cell density and concomitant expression of recombinant proteins can cause several effects that are mainly determined by promoter design and function. The green fluorescent protein (GFP) is an ideal reporter gene for promoter activity studies (Lissemore et al., 2000). Promoter activity can be determined with minimal invasion by measuring emission of light.

Based on their activity profile, promoters can be divided into constitutive promoters, which are active under all culture condition and inducible promoters, which have to be induced by specific factors.

3.2.1 Constitutive promoters

The constitutive promoters of *S. cerevisiae* are most often promoters of house keeping genes mainly derived from the glycolytic pathway, such as *ADHI* (alcohol dehydrogenase) (Ruohonen et al., 1995) or *PGKI* (phosphoglycerol kinase) (Gorgens et al., 2001). The expression of several genes of *E. coli* is also known to be regulated by constitutive promoters (Liang et al., 1999). These genes are highly expressed throughout the whole cell cycle, thus during the complete cultivation time. Constitutive promoters are recommended for the expression of stable and non-toxic products, because induction most often leads to a much lower productivity (Hensing et al., 1995). On the other hand constitutive expression may lead to plasmid instabilities (Wilms et al., 2001) and increases metabolic burden resulting in slower growth. Furthermore, the accumulation of the desired protein can induce stress, which further induces proteolysis or even lysis of the affected cells, especially in HCDF. However, these effects can be avoided by expressing the gene of interest with an inducible promoter.

3.2.2 Chemically inducible promoters

Ideally, induction or de-repression of inducible promoters should lead to strong and efficient transcription of the gene of interest. In its repressed or non-induced state, the promoter should facilitate no or only a low basal expression. Proteins that are toxic or interact with the metabolism of the host should be repressed until induction is desired. The induction agent should be inexpensive in view to large-scale utilization and easy to perform. Classical inducers are substrates that cannot be metabolized, thus providing a long-term induction. This can be achieved by structural analogous as isopropyl- β -D-thiogalactoside (IPTG). This allolactose analogue is used in *E. coli* (Haddadin and Harcum, 2005) as well as in *S. cerevisiae* (Urban et al., 2003). IPTG substitutes the natural inducer and cannot be degraded

by β -galactosidase. However, due to its high costs, toxicity and the formation of inclusion bodies, it is not suitable for HCDF. These detrimental effects of structural analogues can be circumvented by the deletion of genes like the galactose gene. Since such knockout strains cannot metabolize galactose, the sugar can be used as an inducer in *E. coli* (*gal P2*) (Menzella and Gramajo, 2004) and *S. cerevisiae* (*gal1*) (Kang et al., 2005). This technique reduces induction costs 30fold and is thus suitable for HCDF. A strong induction with a factor of 1000 can be achieved.

Deletion of a whole chromosomal gene was used for induction with rhamnose (*rhaB*, L-rhamnulose kinase) (Chae et al., 2000; Wilms et al., 2001). Similar induction is achieved by acetate (Farmer and Liao, 2001) and arabinose (Khlebnikov et al., 2001; Chao et al., 2002) in *E. coli* and sorbitol (*sor1*) (McGonigal et al., 1998) in *S. cerevisiae*. Lactose has been used in *E. coli* (Menzella et al., 2003) and *S. cerevisiae* (Jinzhu et al., 2005). NaCl can also be used for induction. However, its inducing effect is not due to sodium or chloride sensitivity, but its influence on osmolarity (Bhandari and Gowrishankar, 1997). Therefore, the recombinant gene is placed under control of the osmotically inducible *proU* promoter of *E. coli* (Gowrishankar and Manna, 1996) or under the *GDP1* promoter in *S. cerevisiae* (Eriksson et al., 2000). A reduction in the amount of induction chemicals can be achieved by the use of a mammalian steroid hormone for the induction of highly specific hormone receptor. It allows a rapid and strict induction in any yeast species, with very low amounts of deoxycorticosterone (1 nM-100 μ M) (Scheda et al., 1991). Promoters of genes providing heavy metal resistance can further be used for induction (e.g. gold (Stoyanov and Brown, 2003) in *E. coli* or copper (*CUP1*) (Koller et al., 2000) in *S. cerevisiae*).

Many chemical inducers are detrimental, because they interfere with growth or protein biosynthesis. Furthermore, stress induction by osmotic changes can occur and increased downstream effort may be necessary, especially for heavy metals.

The usage of a second non-host related promoter, like the T7 phage promoter in *E. coli* controlled by T7 RNA polymerase, reveals the opportunity to increase the induction efficiency and diminishes the metabolic burden due to competition for transcription factors (Menzella and Gramajo, 2004).

Some chemical inducible promoters also work without the addition of chemicals. These promoters are induced by the depletion of a substrate. *ADH2*, *SUC2* and *CYC1* promoter are derepressed by glucose depletion and *MET25* (Solow et al., 2005), whereas a derepression of the *PISI* (Han et al., 2005) promoter occurs, after methionine or zinc pass a lower limit in *S. cerevisiae*. This might be very useful for HCDF, because stop of influx of fresh media will

effectively induce gene expression. Lee and Da Silva (2005) have shown that the *ADH2* promoter reveals higher applicability for induction of derepression triggered by substrate depletion, compared to the *GALI* and *CUPI* promoters. However, suitability for HCDF is reduced if the feeding strategy is limited by the repressor. Derepression at low repressor concentration and thus unwanted basal expression could occur (e.g. glucose / *ADH2* promoter).

3.2.3 Process dependent promoters

Process dependent promoters are of higher interest than chemically induced promoters. Their induction is easy to perform by changing certain process parameters. Temperature inducible promoters are so far the best studied system. The use of a thermo-labile lacI mutant (Wang et al., 2004) and the strong promoter tandem λ PRPL of the bacteriophage λ (Hoffmann and Rinas, 2000) are used in *E. coli*. The ts-mutation of the regulated GAL promoter of *S. cerevisiae* enables heat induction (Napp and Da Silva, 1994) and allows a tight regulation of the promoters at **higher temperature** (30°C-42°C). Temperature elicited induction is also used in the **cold sensitive** lambda PL phage promoter (Gupta et al., 1999), the cold shock promoter *cspA* derivatives, and the bacteriophage P1 temperature sensitive C1 repressor (Schofield et al., 2002) of *E. coli*. Whereas the promoters PHO5 and operator TPI/a2 are induced through cold shift in *S. cerevisiae* with highest expression observed around 20°C. Cold-inducible promoters were reviewed by Baneyx and Mujacic (2003).

Thermal inducible promoters are cheap, because chemical inducers are not necessary. Moreover induction is easily performed. In HCDF, process heat will increase temperature by several Kelvin per minute by simple interruption of cooling. However, higher incubation temperatures often induces the formation of insoluble inclusion bodies (Schein, 1989), several proteases in the host (Goldberg, 1992) and heat shock proteins that could increase metabolic burden. Cold shift induced processes have the advantage of lower misfolding rate and therefore less formation of aggregates (inclusion bodies). On the other hand, lower temperature decreases the velocity of biochemical reactions, resulting in lower growth and protein production. Nevertheless, temperature reduction can lead to proteins being folded more accurately.

These problems can be avoided by using **oxygen** dependent promoters like VHb (Vitreoscilla hemoglobin promoter) (Khosla and Bailey, 1989; Khosla et al., 1990) and nar (Lee et al., 2003) in *E. coli*, or DAN1 (Sertil et al., 1997) in the yeast *S. cerevisiae*. Oxygen is the major limiting substrate in HCDF. The low water-solubility and the high demand can lead to limitation during cultivation. At this time point, induction is commonly required and easily

achieved by the mentioned promoters. If proper mixing is performed and local limitations are avoided, oxygen dependent promoters can be very useful tools for HCDF.

A third kind of process parameter induced promoters is the **pH-dependent** promoter, like the promoter *cadA* from *E. coli*. During cultivation, pH is maintained by the addition of acid or base, because the uptake or release of pH influencing substrates by cells would change the pH of the media. Short interruptions of pH maintaining additives during HCDF, similar to heat induction, will result in strong pH shifts usable for the induction of pH dependent promoters in HCDF (Chou et al., 1995).

The usage of stress inducible promoters, such as a promoter from *Marinococcus halophilus* for *E. coli* (Bestvater and Galinski, 2002) or light inducible promoters, such as PIF3 (Shimizu-Sato et al., 2002) used in *S. cerevisiae* are not of high value for HCDF, because high cell density represents stress and light can not permeate the culture medium.

3.2.4 Growth phase dependent promoter

Promoters that are induced by cell conditions could play a significant role in the future of HCDF. Stationary phase promoters of *E. coli* were analyzed by Shimada et al. (2004). Induction at transition from logarithmic to stationary phase would lead to highest biomass and strong induction to promote high protein expression. These kinds of promoters are applicable for secondary metabolites or products that would interact or inhibit primary metabolites.

3.2.5 Conclusion of promoter

A variety of different promoters have been discovered and applied for heterologous gene expression. An increasing number of different promoters reveal the opportunity to choose the kind of induction that is suitable for a certain problem. Both hosts *E. coli* and *S. cerevisiae* (Zhu and Zhang, 1999) possess an assortment of different promoters that can be successfully applied. Further promoters can be found in other species and have already been adapted and applied in these hosts. However, not all of these promoters can be used in HCDF. Nonetheless, usability could be increased by improvements of process and reactor design could also make so far unsuitable promoters available. Moreover, the de novo generation of strong promoter elements by error prone PCR of DNA sequences without promoter activity offers a new approach for the generation of promoters with properties of certain interest (Kagiya et al., 2005).

4 Process development

A definition of HCDF is necessary and the question of the highest achievable dry cell weight has to be answered, before the process design is discussed. Although the definition of HCDF seems to be very simple, the threshold value is still subject to discussion. Some publications deal with approx. 20 g dry cell weight per liter (gDCW l⁻¹) of an *E. coli* fermentation broth (Li et al., 2000). However, this value is much lower for eukaryotic cells. The maximal achievable biomass depends on the cultivated organism and its metabolism. It was calculated to 400 gDCW l⁻¹ (whole reactor packed with cells; 20-25% dry cell weight per wet weight; V=0.4 x 2 μm) (Riesenberg, 1991). This theoretical value cannot be reached because of strong mass transfer limitations in a reactor which is almost entirely filled with cells. However, this value is reduced to 200 gDCW l⁻¹ for an *E. coli* cultivation, if more realistic parameters are used within this calculation (Markl et al., 1993). This value has been accomplished for the production of polyhydroxybutyrate (Choi and Lee, 1999). Moreover, a yield of up to 20 g l⁻¹ of recombinant protein could be produced, if 20% of total protein were recombinant proteins (Panda, 2003). High protein concentrations and the production of toxic by-products limit the obtainable maximal biomass concentration. Each of these factors is strongly host dependent, which makes it nearly impossible to compare different hosts and even different strains. A dry cell weight of 100 g l⁻¹ is a value that is in achievable range for a cultivation of prokaryotes and simple eukaryotes.

4.1 Types of cultivation

Since the cultivation of both hosts *E. coli* and *S. cerevisiae* is quite similar, the following discussion on process development will not focus on a certain microorganism but look at general properties. Cultivations of both hosts can be achieved by different types of fermentation. Batch fermentation is a closed system, which includes all growth substances. Once substrate is almost entirely assimilated, the fermentation broth is harvested and the product is recovered either from medium if secreted or from the cells if expressed intracellular. Batch fermentations are preferred at industrial scale, because of their variability and easy planning. The more complex continuous fermentation is performed at constant culture volume. This is achieved by a continuous addition of fresh medium to the vessel while culture broth is harvested. Growth of cells is restricted by the feeding rate, because of limitation of a chosen substrate. Once a steady state is reached, the concentration of nutrients remains constant, which is a big advantage of continuous fermentation. Despite its higher productivity, the number of application in industrial production is low, because of high

process stability requirements, contamination risk and complex automation technique. A compromise between the two fermentation techniques mentioned is the so called “extended batch” or “fed-batch” process, where initial batch fermentation is prolonged by stepwise or continuous addition of fresh media. The use of computer controlled feeding allows cultivations without accumulation of strain dependent toxic byproducts, such as acetate for *E. coli* (van de Walle and Shiloach, 1997) or ethanol for *S. cerevisiae*. Moreover, feeding of substrates that may be inhibitory at higher concentrations is possible. Achieved biomass yield and product formation can be higher, compared to batch or continuous cultivation, which makes fed-batch cultivation the preferred and inevitable type for achieving HCDF.

4.2 Feeding strategies

4.2.1 Feeding without feedback control

Fed-batch fermentations can be performed either with feedback control or without such control system. The latter can be divided further into feeding with **constant feeding** rate, increased feeding rate and predetermined exponential feeding. Constant inflow of fresh media results in a constant decrease of specific growth rate (μ) and product formation rate (π). High process stability and low accumulation of by-products are advantages. However, long cultivation times and low productivity are disadvantageous (Prytz et al., 2003). **Increased feeding rate** is performed by stepwise or linear increase of inflow of fresh media. A decrease of specific growth rate (μ) can be compensated partially and HCD conditions are achieved earlier, but the risk of toxic byproducts is higher. **Predetermined exponential feeding** (Fan et al., 2005) reduces the by-product formation and the fermentation time by keeping μ constant. However, declining process stability is a big drawback of this feeding profile. Nevertheless, the feeding without feedback control has the advantage of lower equipment requirements and failure of measurements cannot influence feeding rate.

4.2.2 Feeding with feedback control

The feeding with feedback control is a growing field, because identification of new measurements of the actual culture state will introduce new feeding strategies. Different parameters can be used to control subsequent feeding. Increasing oxygen saturation indicates substrate limitation and is used as parameter for **DO-stat feeding** (Whiffin et al., 2004; Oliveira et al., 2005) in defined medium, whereas pH changes, due to depletion of primary C-sources, is used for **pH-stat feeding** (Porro et al., 1991) in complex medium. **Carbon dioxide evolution rate** (CER) uses mass spectrometer and cell concentration to calculate the

remaining C-source and the necessary feeding rate (Nilsson et al., 2002). Moreover, feeding control by direct measurement of **cell concentration** through laser turbidimeter is possible. Optical measurements are of high interest because of their robustness and non-invasive nature. Besides turbidimeter measurement, fluorescence (Sundaram et al., 1997), near-infrared (Blanco et al., 2004) and in situ microscopy (Bittner et al., 1998) are in use. Most recently, Hofmann et al. (2005) presented a transponder based sensor for monitoring electric properties correlating with optical density.

The **combination** of the former mentioned control systems is also possible. The possibility to use predetermined exponential feeding and interruption of feeding in case of pH-change was reported by Kim et al. (2004). It diminishes the instability of predetermined exponential feeding mentioned above.

The feedback control gives the possibility to control feeding by measurement of essential parameters. If the measurement is reliable, a stabilization of the whole process can be obtained. A major drawback is the necessity of expensive equipment, such as mass spectrometer. However, at industrial scale these costs are compensated by increased yield, productivity and process stability.

Feedback controlled feeding is not always advantageous compared to predetermined feeding profile. In the case of methanol feeding to *P. pastoris* culture the limited **predetermined exponential feeding** profile resulted in a higher specific productivity due to the lower methanol concentration (Trinh et al., 2000; Zhang et al., 2000). Artificial neural networks and fuzzy logic are used as software sensors if information is sparse or deterministic models are not available.

4.2.3 New sensors for process control

Process control is given by a reaction of an executive device to certain process parameters. Therefore, the access to those parameters is of major interest. However, the lack of robust and reliable sensors is a major burden for the control of bioprocesses. Some sensors are known since decades. Nonetheless, they are continuously refined, like the **ethanol sensor**, which is used for direct control of recombinant HCDF in *S. cerevisiae* (Noronha et al., 1999). The operation extension of known sensors presents another way to receive new process parameter. One example is the oxygen sensor, which can also be used for acetate measurement (Åkesson et al., 1999). Furthermore, online estimation of biomass and acetate formation by measurement of **base consumption and conductivity** (Hoffmann et al., 2000) as well as the determination of the fitness of *E. coli* strains during HCDF by measuring extracellular cAMP-concentration (Lin et al., 2004) are reliable tools for HCDF. Additionally, Hewitt et al. (1999)

and Zhao et al. (1999) have reported the use of **flow cytometry** to characterize the physiological state during fermentation and to influence process parameter.

Reporter genes, such as the green fluorescence protein (GFP), present another alternative to determine certain process parameters, which can be used to optimize product yields. If GFP is under the control of **stress** promoters, such as HSP12 from yeast (Karreman and Lindsey, 2005), fluorescence measurements can be used to determine stress in relation to cultivation parameters.. Process related stress in large bioreactors were further analyzed by Schweder et al. (1999). Chambers et al. (2004) expressed the GFP gene under the control of the **diauxic shift** promoter JEN1 from *S. cerevisiae* and could determine glucose concentration at steady state by fluorescence measurement. Fused with the recombinant product, GFP can be used for direct observation of recombinant protein production and gives the opportunity to optimize recombinant protein expression preliminary to large scale fermentations (DeLisa et al., 1999; Chae et al., 2000). Beside physiological conditions, molecular parameters are also of growing value. Most recently, new approaches for analyzing complete sets of RNA or protein levels reveal a deeper insight into the status of cells in HCDF. Such **transcriptome and proteome analysis**, even if not used for online control, can reveal conditions, which negatively affect yield (Yoon et al., 2002; Baev et al., 2006). Since the first response of cultivated cells on certain conditions occurs on the RNA-level, the online measurement of this parameter allows a much earlier intervention in a running process. The control of fermentation by computer gives the opportunity to freely configure control circuits and permit remote access to the fermentation system over the internet (Kellerhals et al., 1999). Developments in the application of new control systems are reviewed in an excellent and detailed manner (Riesenberger and Guthke, 1999).

4.3 Cultivation medium

The most important factor to achieve HCDF is the composition of the cultivation medium. Its importance for cell growth as well as its influence on the costs of the entire process were addressed by a number of publications concerning culture medium and medium components (Gabbrakhmanova et al., 1996; Kadam and Newman, 1997; Aristidou et al., 1999).

The composition of the cultivation medium has to be sufficient for product formation and unlimited growth. However, a growth inhibitory accumulation has to be avoided. This is lowered by continuous feeding and control of supernatant composition. Continuous cultivation, as already mentioned above, is not commonly used for industrial purposes so far. Nevertheless, it is a useful tool to investigate the nutrition requirements of a culture (Larsson et al., 1993). Determination of nutrition requirements will be performed over several

generations and at different dilution rates to ensure long time cultivation condition. It is also employed to determine the reaction of cells to different feeding rates. The optimal feeding rate for subsequent fed-batch cultivation can be determined graphically from the X-D diagram, which shows the accumulation of biomass against the dilution rate. Dilution rate dependent changes of cell composition can be analyzed similarly (Ertugay and Hamamci, 1997). Beside this, product formation can not only be monitored, but correlated to the dilution rate. Kinetic parameters, such as product formation rate (π), maximal specific growth rate (μ_{\max}) and the biomass to substrate yield ($Y_{X/S}$) can be calculated. In this context, $Y_{X/S}$ represents a variety of different values, because a specific value can be calculated for each substrate, e.g. for carbon. Therefore, preliminary cultivation of a certain strain in continuous culture will generate important knowledge in order to develop a successful HCDF.

The concentration of the cultivation medium to be fed should be as high as possible in order to prevent dilution and decreased volumetric productivity. From this point of view, the solubility of the components becomes very important, because precipitations have to be prevented. This can be achieved by adding chelating agents like Na-citrate or EDTA. Moreover, the pH can be adapted to raise the solubility of complex N-sources. The use of new supplements, such as polyphosphate glass (sodium hexametaphosphate) as a source of phosphate in HCDF can increase concentration (Curless et al., 1996). Precipitations are prevented, since it is highly soluble and does not form metallophosphate precipitates.

Osmolarity is another factor, which is dependent on the concentration of the medium. It influences cell growth and product yields. If a medium with high substrate concentration is used, lag-time can be shortened and leavening ability can be increased by a pre-treatment of the cells in hyperosmotic media (Hirasawa and Yokoigawa, 2001). The use of osmoprotectants, such as proline or glycine betaine can also positively influence viability and yield (Thomas et al., 1994).

Oxygen is the most important substrate for achieving HCDF. Its solubility in water is very low, which limits the available oxygen concentration. Since HCDF have a very high oxygen demand, continuous feeding and measuring is necessary to ensure an optimal oxygen supply of the cells. Oxygen limitation is one of the most detrimental effects. It is enhanced by increasing the viscosity through higher biomass concentration. Different strategies can be applied, to overcome or prevent an insufficient oxygen supply. At first, air inflow and stirring speed are increased, to improve specific volumetric area (kLa). Silva et al. (2002) have shown, that these parameters influence the protein production. Furthermore, a decrease of temperature will lead to a decreased oxygen demand and an increase in oxygen solubility.

Finally yet important, oxygen enriched air can be used for aeration of HCDF. If the use of pure oxygen is prevented or the impeller speed is not increased due to shear stress, the hydrostatic pressure can be increased in the reactor to reach the desired partial pressure of oxygen. This method becomes especially important in reactors without stirrer, such as air lift fermentor (Klein et al., 2005). Hyperbaric fermentations can be carried out for several microorganisms up to 10bar (1.0MPa) if a proper pressurization mode is used (Belo et al., 2003). Most recently, Garcia-Ochoa and Gomez (2005) successfully performed the prediction of gas-liquid mass transfer coefficient for different systems. However, a very high oxygen concentration has to be prevented, since it can negatively affect yield by changing the oxidative level of enzymes. Furthermore, cost factors for energy and oxygen supply have to be heeded. Hydrogen ions in solution, measured as pH, represent a second important substrate. Many enzymatic reactions are pH dependent, and enzymatic reactions may vary at different pH ranges. Moreover, degradation of secreted recombinant protein is dependent on pH and medium composition (Kang et al., 2000), which suggests that pH-dependent proteolysis may occur.

Last but not least, the optimal temperature of a cultivation, which is the temperature of highest growth, is an important factor in HCDF. Chemical reactions are faster at higher temperatures, but above a certain temperature a decrease in growth is observed due to a heat inactivation of temperature labile enzymes. Metabolic burden through expression of heat shock and plasmid related proteins increases significantly by temperature shift induction. This leads to a decrease in growth and biomass yield (Hoffmann et al., 2002). However, biomass yield may not be optimal at the “optimal growth temperature”. Some examples are known, where optimal growth temperature is related with lowest protein and RNA yield (Verduyn, 1991). Yet, different species show different behavior.

5 Conclusion

The achievement of HCDF depends on a variety of parameters. The majority of these are host related or directly depend on strain properties. As represented, prokaryotes and simple eukaryotes were successfully cultivated to very high cell concentrations. In order to produce high yields of recombinant proteins, the specific protein production as well as the cell density has to be increased. The first is primarily achieved by genetic manipulations, whereas the second is achieved by process design. Both ways are indispensable and benefit from each other. The long procedure of strain seeking, selection, improvement and accompanied process adaptation can be circumvented, if a completely characterized working strain is established. With the help of genetic and process related information it will be possible to create a strain

that is highly suitable for cultivation and recombinant production. If this host shows all the important properties, it can be used for every biological product. The classical production will change to a process, where the production strain and the process will mutually give assistance to reach highest yield of recombinant product. It will not be necessary to select between different organisms and strains. The existent production strain will fulfill all requirements for efficient recombinant production. Further knowledge of genomic information and the discovery of new genetic methods will enlarge the spectrum of biotechnological products that can be produced with already employed production strains.

II. PRACTICAL PART

Heterologous expression and purification of wheat storage proteins in the yeast

Saccharomyces cerevisiae

1 Introduction

1.1 Project information

The presented work is part of the „Leitprojekt - Entwicklung von Weizen-, Roggen- und Gerstenproteinen ohne Zöliakietoxizität und deren Verwendung zur Herstellung von Lebensmitteln“, which raised from the „Leitprojektinitiative Ernährung – moderne Verfahren der Lebensmittelerzeugung“, of the “Bundesministerium für Bildung und Forschung” (Förderkennzeichen 0312246C).

Main aim of the project was the identification of the wheat storage proteins, which show no celiac toxicity. The separation of pure single WSPs cannot be achieved so far, because of the covalently developed network and the high homology in structure and sequence of the different subunits. Determination of toxic regions is not possible, because of contamination with homolog subunits, when directly purified from wheat. The expression of single WSPs in *S. cerevisiae* provides a tool to gain purified products without these contaminations. Therefore, the identification of toxicity becomes reliable and cross reaction can be excluded.

One further main aim of the project was the production of food that shows no celiac toxicity. This would be possible by the addition of wheat storage proteins extracted from genetically modified yeasts to flour without celiac toxicity (e.g. maize). This should result in baked goods with baking characteristic and sensorial and nutritional properties similar to the known products.

1.2 Background of the project

1.2.1 Celiac disease

Celiac disease, which is also called c(o)eliac sprue, non-tropical sprue, gluten enteropathy, and gluten intolerance, is an incompatibility reaction against gluten protein that is found in wheat, rye, and barley. That oat does not show the formerly reported celiac toxicity ([Lundin et al., 2003](#); [Peraaho et al., 2004](#)) was found recently ([Holm et al., 2006](#); [Srinivasan et al., 2006](#)). The clinical picture shows vomituration, diarrhea and scarcity symptoms, which is combined with an inflammable degradation of the intestine villi and thus with a reduction of the

resorption surface. The breakout of this disease in genetically predisposed persons is triggered by prolamins (alcohol soluble storage proteins). For the avoidance of the disease respectively their symptoms affected persons are dependent on a lifelong gluten free diet. This diet not only contains the avoidance of cereal products as bread, pasta or even beer, but also products, whose rheology is changed by the addition of such cereal proteins. The risk of intestinal cancer is strongly increased if the strict diet is not maintained (Fasano et al., 2003). The huge number of up to 300.000 affected humans in Germany, which suffer of a considerable restriction in their quality of life, is reflected by the labeling obligation of gluten containing food.

1.2.2 Wheat storage proteins

WSP are located in the endosperm of the wheat grain and consist of approximately 80% of the total protein. This reservoir of nutrition for the growing embryo represents matrix-proteins, which give wheat flour the property to form dough that has high gas retention ability and contributes to the best baking property compared to other cereal varieties. Wheat gluten consists of up to 90% proteins (Figure 3), 8% lipids and 2% carbohydrates (Belitz et al., 2001).

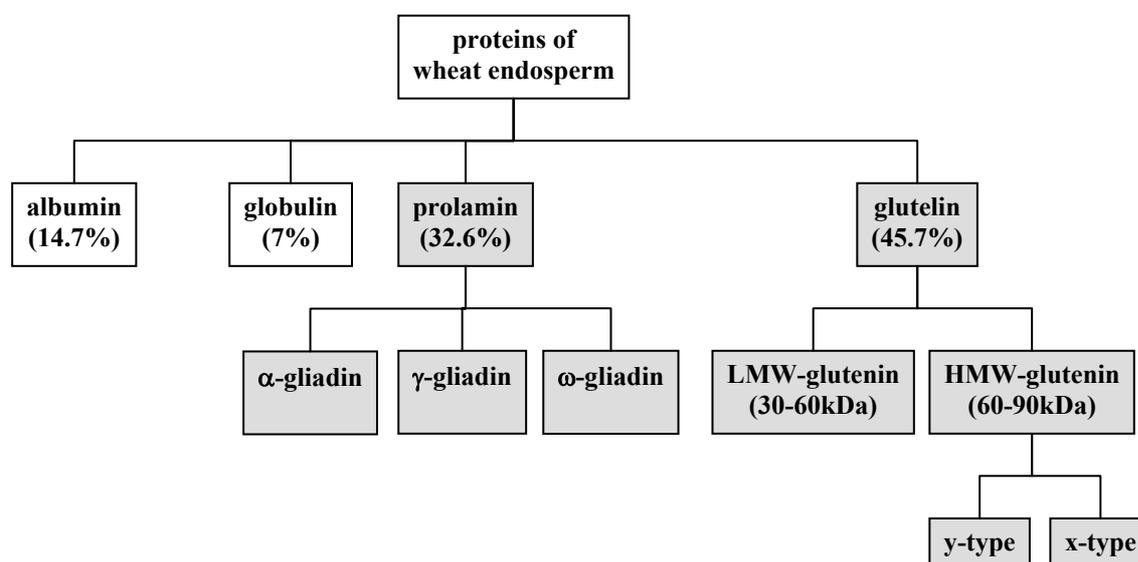


Figure 3: Proteins of the wheat endosperm (Ternes, 1993)

The classification of the WSP was firstly performed by Osborne et al. (1907) through the fractionated solvation. The so called “Osborne-fractions” are divided in the water soluble albumins, the salt soluble globulins, the alcohol soluble prolamins that can be extracted with 70% ethanol and the insoluble glutelins (Figure 3). Glutelins can be extracted in alcoholic solutions with reductive agents and raised temperatures. With the help of electrophoretic and

chromatographic procedures the polymeric WSP can be separated by their molecular weight into the high molecular weight subunits (HMW) and the low molecular weight subunits (LMW). They consist of cystein residues, which are responsible for the formation of intermolecular disulfide bridges. The wheat storage protein related monomeric prolamins were divided by its molecular weight and its hydrophobicity into α -, β -, γ - and ω -gliadins. First three are rich in cysteines and are able to form intramolecular disulfide bridges. The ω -gliadins are poor in cysteines and therefore form only a small amount of intramolecular disulfide bridges. Gliadins and glutenins (in wheat), secalins and secalinins (in rye) and hordeins and hodenins (in barley), respectively, represent the storage proteins that are supposed to mediate celiac toxicity in the certain cereals.

1.2.3 Basics of the two-phase formation

Aqueous two-phase systems are used since decades to isolate biological molecules and particles. They are based on incompatibility of polymers or mixtures of polymers and salts. The high number of parameters, which influences the partition of biomolecules within the different phase, makes it impossible to predict the concrete distribution. Knowledge of the properties of the certain product can help to estimate partition. However, only some rules of thumb are known that meet practical application. The direct influence of partition parameters can only be examined empirical. Hints for the certain strategy and a good introduction into the topic ATPS are given by the series “Methods and Protocols” (Hatti-Kaul, 2000).

Three different kinds of phase systems are known, which are divided by the phase forming component. First and most investigated system is the polymer-polymer-system. They are not used for industrial purification, because of the high price of polymer components. The second kind of systems, which came in focus in recent time, is the thermoseparating system. They consist of only one polymer, usually a detergent (e.g. Triton X114). Two phase separation is induced by temperature increase, which changes water solubility of the polymer. The third and most prominent systems are the polymer-salt-systems. They are commonly used for industrial purposes, because of its low costs, fast separation and safe phase forming compound. PEG/phosphate-systems are widely used and will also be employed for the separation of the recombinant expressed WSPs.

Commonly, the separation of the aqueous phases takes place in two working steps: Firstly, the adjustment of equilibrium and secondly the separation of the phases. The equilibrium is caused by strong mixing of the phase components. The separation by ATPS needs more time than with organic two phase systems (OTPS), because of the small density differences of the phases, the higher viscosities and the development of aggregates. The separation can be sped

up by centrifugation and other methods. Phase mixing and cell disruption can be applied simultaneously (Su and Feng, 1999).

The high hydrophobicity of the product increases the suitability of ATPS, because of specific partition in special systems (Andrews et al., 2005). Although the extraction of a WSP by OTPS could be achieved (Truust and Johansson, 1998), ATPS have some advantages in contrast to OTPS. Organic extractions are incompatible with conformation stability. The main component of ATPS is water. This forms a mild environment for sensitive biomolecules like enzymes. The surface tension of ATPS is lower in comparison with water-solvent-systems. Additionally, it is known that polymers have a stabilizing effect on the biological activity of biomolecules (Albertsson, 1986). Furthermore, the proteins are extracted in native form instead of reduced or denatured. Nevertheless, the mass transfer is also immeasurably rapid in ATPS.

1.2.4 Starting point

Starting point of this work was several recombinant *S. cerevisiae* strains that carried vectors or integrative constructs. Vectors were episomal plasmids that were derived from the naturally occurring 2 μ m-plasmid in *S. cerevisiae*. The respective constructs carried following genes: HMW-1Dx5 and HMW-1Dy10 from *Triticum aestivum* (Cheyenne variety), which are correlated to good baking quality (Altpeter et al., 2004) and carried no known celiac toxicity potential. LMW 6 from *Triticum aestivum* (Cheyenne variety) represented a LMW-gene that consists of no known toxic or allergic epitopes. The LMWa3-gene from *Triticum aestivum* (Florida variety) worked as a gliadin replacement, because of its structural homology and PCR-aided mutation of two cystein residues, by this it was transferred to a monomeric protein (Hinzmann et al., 2002).

1.2.5 Aims of the thesis

The major aim of this work was the production of recombinant WSPs in sufficient amounts for the subsequent determination of the toxicity of the single wheat storage protein subunits and the establishment of a cultivation process with respect to large scale. Productivity of such a process can be increased in two ways: by the increase of specific protein production and by the decrease of protein loss during purification.

The increase of recombinant protein yield can be achieved by genetic and process related factors (see “Theoretical Part”). This work focused mainly on the process related factors. At the beginning, medium optimization in shake flasks was performed to improve biomass and product formation. Afterwards, process parameters, which were hidden in shake flask

experiments e.g. pH value, oxygen transfer, feeding strategies and suitable limitations, were optimized in larger scale.

The prevention of loss of specific protein was achieved by the adaptation of the Osborne-fractionation to yeast lysate. The investigation of parameters, which quantitatively influence the product recovery at alcoholic extraction, could lead to higher overall recombinant protein output, reduction of chemicals and higher product purity. However, alcoholic extraction needs very drastic conditions as high temperatures, addition of non-polar solvents and the addition of reducing agents. For the production of food these conditions represent, however, a possible health risk.

An alternative extraction represents the aqueous two-phase extraction. Here food-suitable substances as polyethylene glycol (PEG) and simple salts (e.g. potassium phosphate or ammonium sulfate) are used. Its simple feasibility and scale up as well as the high biocompatibility make ATPSs attractive for technical bio-separations.

1.2.5.1 Summary

- Increased protein- and biomass-yield through:
 - Medium optimization
 - Optimization of fermentation parameters as pH, temperature, aeration, feeding or suitable limitations
- Increased protein yield through:
 - Adaptation of the classical Osborne-fractionation
 - Establishing of an aqueous two-phase extraction

2 Materials and methods

2.1 Devices

Autoclave:	165 l (Fedegari-Pavia)
Balances:	Type 1907 and Type 2462 (Sartorius)
Cell desintegrators:	FastPräp FP120 Bio101 (Thermo Savant), Biomatik-Celldesintegrator-S (Biomatik GmbH), DynoMill (W. A. Bachofen)
Centrifuges:	Sorvall RC5C and Sorvall RC2-B (DuPont); Mikrorapid/K, Mikro 22R and Rotina 35 R (Hettich)
Current supply:	Phero-Stab 500 (Biotec-Fischer); Power~Pack 300 (Bio-Rad)
Dry blotter:	Semidry-blotter (H. Hölzel)
Electrophoresis chamber:	Mini-Protean® 3 Cell (Bio-Rad); Mini Sub™ DNA cell and wide mini Sub™ cell (Bio-Rad), Horizontal electrophoresis chamber (AGS, Heidelberg)
Fermentor:	Biostat E; 2.5l and 10l vessel (B. Braun)
Gel documentation:	Kodak image station 440 CF (INTAS)
Gel drying films:	Gel drying film (Promega Corporation)
Hybridization oven:	Mini oven MKII (MWG-Biotech)
Incubator:	Type 5050 (Heraeus)
Microscope:	Biomed (Wildt Leitz GmbH)
PCR-device:	T-gradient whatman (Biometra); T3 thermocycler whatman (Biometra)
pH-detection:	Digital pH-meter 646 (Knick)
Pipettes:	P10, P20, P200, P1000 (Gilson)
Pressure Blot Apparatus:	PosiBlot 30-30 pressure blotter (Stratagene GmbH)
Rotors:	SS34, SLA1500, GSA, fixed angle rotors (DuPont); 1015, 1158, fixed angle rotors (Hettich); 1713, swing out rotor for microtiter plates (Hettich)
Spectrophotometer:	Uvikon XS with thermopack (Bio-Tek)
Sterile bench:	uvub 1200 (Uniflow)
Ultrasonic basin:	Sonorex TK52 (Bandelin)
Ultrasonic tube:	Braunsonic 300S (B.Braun)
UV-crosslinker:	UV Stratalinker™ 1800 (Stratagene)

UV-transilluminator:	Intas TF-35-C ($\lambda_{\text{Fix}} = 254 \text{ nm}$), (Vilber Lourmat)
Video documentation:	Intas (Vilber Lourmat)
Water bath:	GFL-Type 1083, RM6 (Lauda), Thermomix 1460 (B. Braun)
X-ray exposure:	Kodak X-ray exposure holder

2.2 Enzymes, kits and chemicals

2-propanol:	Roth
Chemo luminescence substrate:	SuperSignal [®] West Pico (Pierce)
Chromatography-paper:	Whatman, 3MM Chr
GeneRuler [™] , Taq Polymerase:	MBI Fermentas
Hybond N membrane:	Amersham-Buchler GmbH
Nitrocellulose-Blotting-Membrane:	Sartorius
Nitrogen:	Linde, technical gas
NZ-Amine A:	Sigma
Oxygen:	Linde, technical gas (99.8% oxygen)
PMSF:	Fluka
Tris, glycerin:	Sigma
Tryptone:	Oxoid
Sorbitol:	Roth
Roentgen films:	Super RX Medical X-RAY Film (Fuji)
Roentgen film developer:	Kodak
Western blot antibody I (LMW):	Monoclonal antibody PN3 (Ellis et al., 1998); kindly supplied by H. J. Ellis, Gastroenterology Unit (UMDS), St. Thomas's Hospital, London UK
Western blot antibody I (HMW):	Monoclonal antibody IFRN 1602 (Mills et al., 2000), kindly supplied by E. N. C. Mills, Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA UK
Western blot antibody II:	Rabbit anti mouse immune globulins HRP (DAKO)

Not listed substances or products were supplied by Serva, Merck, Roche, MBI Fermentas or Sigma. All chemicals were of analytical grade or higher.

2.3 Cultivation related methods

2.3.1 Strains

Saccharomyces cerevisiae:

- AH22 Δ *pmt1* Δ *prb1*: AH22*ura3*-Derivat Δ *pmt1*, Δ *prb1* (Hinzmann, unpublished)
- AH22*ura3*: AH22 (*MATa*, *leu2-3*, *leu2-112* *his4-519*, *can1* (Hinnen et al. 1978))-derivative with additional deletion of the *ura3*-Gens (Polakowski 1999)
- GRF18: *MATa*, *leu2-3*, *leu2-112*, *his3-15*, *can1*, *mal* Institut für Gärungsgewerbe, Berlin, (IfGB 07221)
- GRF18*ura3*: GRF18-Derivat with additional deletion of *ura3*-gene (Veen, in progress)
- Mü4WSP: mated diploid strain of AH22 Δ *pmt1* Δ *prb1* HMW-1Dy10 HMW-1Dx5 and GRF18 Δ *pmt1* LMW6 HMW-1Dx5 YpADH Δ AIAQ-LMWa3 Δ *cys2-leu2d* (Müller, PhD thesis 2006). Integrative constructs were obtained by homologous recombination into the 420 free or Ty-element related δ -elements, flanking regions of retro-transposons of the yeast.

The Strain GRF18 was kindly supplied by B. Prinz, GRF18*ura* by M. Veen, Mü4WSP by M. Müller and AH22*ura3* as well as AH22 Δ *pmt1* Δ *prb1* by E. Hinzmann.

Plasmids:

- YpADH-LMW6 YpADH-URA3-derivative carrying a PCR-fragment of LMW6 gene from genomic DNA from wheat
- YpADH-LMWa3 Δ *cys2* YpADH-URA3-derivative with PCR-fragment of the modified LMWa3 Gene, *Cys1* and *Cys7* mutated – Patent: (Hinzmann et al., 2002) derived from the plasmid pBluescript
- YpADH-HMW 1Dx5 YpADHsec-derivative with URA3- and HMW-1Dx5 gene from p57
- YpADH-HMW 1Dy10 YpADHsec-derivative with URA3-Gene and HMW-1Dy10 Gene from p2-44

YpADH-LMW6, YpADH-LMWa3 Δ *cys2*, YpADH-HMW-1Dx5 and YpADH-HMW-1Dy10 were kindly supplied by E. Hinzmann.

2.3.2 Media, culture and storage conditions

S. cerevisiae:

YEED-Medium: complex medium (20 g l⁻¹ peptone, 10 g l⁻¹ yeast extract, 20 g l⁻¹ glucose)

YE-Medium: complex medium (5 g l⁻¹ yeast extract, 20 g l⁻¹ glucose, pH 6.3)

YNB-Medium: minimal medium (6.7 g l⁻¹ yeast nitrogen base w/o amino acids (Difco), 20 g l⁻¹ glucose, if necessary leucine (0.4 g l⁻¹), histidine (1 g l⁻¹), uracil (0.4 g l⁻¹), NZ-Amine (NZ, 0.2 g l⁻¹) were added.

DS-Medium: Synthetic medium (50 g l⁻¹ sucrose, 10 g l⁻¹ sodium-glutamate x H₂O, 5 g l⁻¹ (NH₄)₂SO₄, 3.2 g l⁻¹ NH₄H₂PO₄, 1.5 g l⁻¹ KCl, 0.75 g l⁻¹ MgSO₄ x 7 H₂O, 0.1 g l⁻¹ CaCl₂ x 2 H₂O, 0.1 g l⁻¹ myo-Inositol, 5 g l⁻¹ casamino acids (CA), 4 ml l⁻¹ vitamin solution, 4 ml l⁻¹ trace element solution, 2 ml l⁻¹ iron sulfate solution

Iron sulfate solution 2.0 g l⁻¹ FeSO₄ x 7 H₂O

Trace elements solution 6.98 g l⁻¹ ZnSO₄ x 7 H₂O; 0.4 g l⁻¹ CuSO₄ x 5 H₂O;
0.4 g l⁻¹ NaMoO₄ x 2 H₂O; 6.98 g l⁻¹ MnSO₄ x H₂O

Vitamin solution 11.56 mg l⁻¹ biotin, 2.036 g l⁻¹ calcium pantothenate,
2.0 g l⁻¹ pyridoxine, 2.0 g l⁻¹ nicotinic acid, 2.0 g l⁻¹
thiamine

For surface cultivation in dishes 15 g l⁻¹ agar was added.

Cultivation: 28°C 1-2 days, 20 - 250 ml shake flasks were shaken at 120 – 160 rpm

Storage: all strains were stored at -70°C in 30% (v/v) glycerol

2.3.3 Fermentation

2.3.3.1 Preculture

Preculture was performed in two steps. 20ml medium containing 100ml shake flasks were inoculated from frozen glycerol stocks and cultivated at 120 rpm and 28°C on horizontal shaker. Second preculture was inoculated from first cultivation to OD 0.2 in 500 ml baffled shake flasks containing 100 ml medium on circular shaker at 120 rpm. Numbers of second precultures were adapted to the required biomass amount.

2.3.3.2 Batch, fed-batch and continuous culture

2l and 10l fermentors with the possible in place sterilization were used for cultivation. Central processing unit was a Biostat E (B. Braun, Melsungen, Germany) with measure and control units for temperature, pH value (pH-electrode, Fa. Mettler Toledo, Switzerland) and dissolved oxygen (pO₂-electrode, Ingold, Zurich, Switzerland). Process data as temperature (°C), dissolved oxygen (% saturation), pH value, stirrer speed (rpm), acid/base consumption (seconds of titration cycle) were recorded by a plotter. The medium was sterilized without heat labile substances. Before inoculation took place, heat labile substrates were added and volume was controlled or reset. The pO₂-electrode was polarized for at least 12 h and calibrated with nitrogen and air. The pH-electrode was calibrated with appropriate buffers before sterilization occurred and correct measurement was controlled via sample pH. Conditions were set as desired and batch fermentation was started through inoculation, when parameters were correct. At the end of the batch phase, harvesting or start of fed-batch or continuous cultivation was performed. The volume for continuous culture was kept constant by level adjustment through removing excess by pumping. Feed media were sterilized in 10 l-bottles without heat labile substrates. Before connection over coupling, the feed medium was provided with the labile substrates and well mixed. Following conditions were kept constant in almost all fermentations:

temperature:	29°C
pH-value:	4.5 - 5.5
aeration:	1 vvm

Adjustment of pH was achieved through addition of 25% NH₃ (or 4 M (NH₄)₂SO₄) and 4 M H₂SO₄. Dissolved oxygen was kept above 20% by manually increase of impeller speed and aeration. After impeller speed and aeration reached its maximum, 99.8% oxygen was applied to achieve sufficient oxygen saturation. Addition of antifoam was manually performed, when foam sufficient foam was produced. Sample taking was achieved through the bottom valve (10 l vessel) or through sample pipe (2 l vessel). Both ways showed low dead volume. The fermentor is represented in Figure 4.

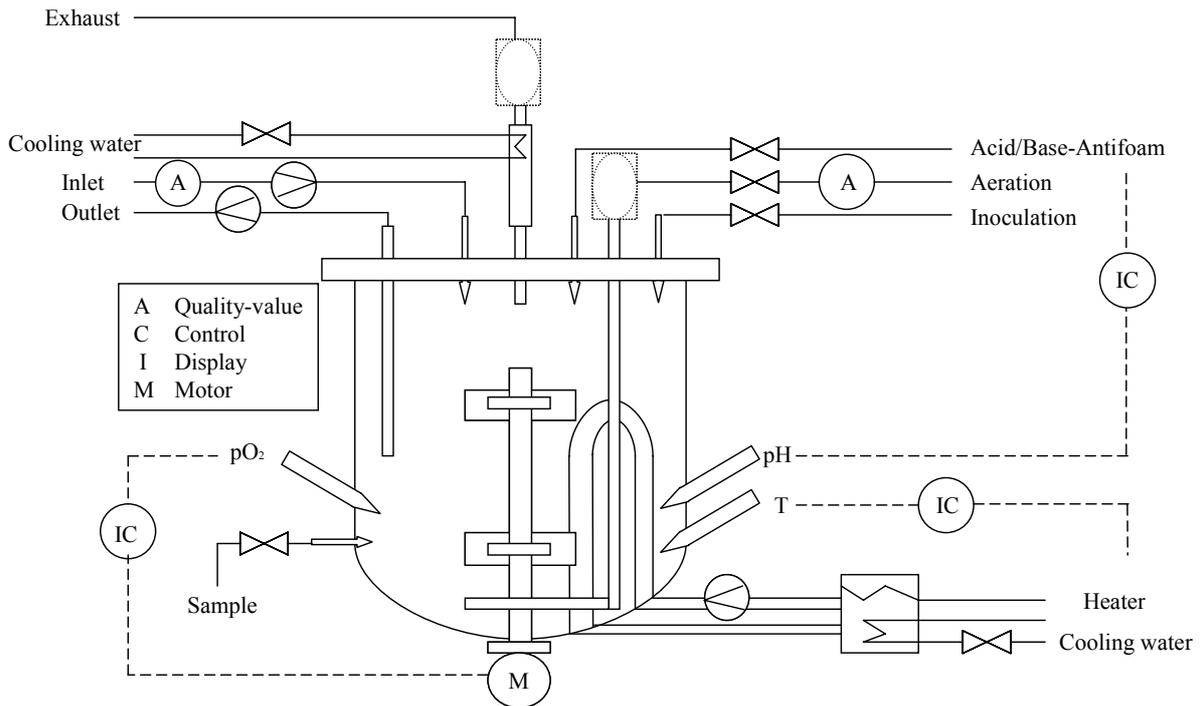


Figure 4: Schematic sketch of the used fermentor unit

2.3.4 Determination of cell number

Cell number was determined in a Thoma-chamber ($0.0025 \text{ mm}^2 \times 0.1 \text{ mm}$) and following equation:

$$\text{cellnumber} \times \text{ml}^{-1} = \frac{\varnothing \text{ZN} \times 10^6 \times \text{DF}}{4} \quad (1)$$

$\varnothing \text{ZN}$ means the average cell number from four quadrates in the chamber and DF means the dilution factor.

2.3.5 Determination of optical density

Optical density (OD) was measured at $\lambda = 600 \text{ nm}$ in duplicates with culture medium as reference. Calculation took place according to Lambert-Beer.

2.3.6 Determination of medium components

Sucrose, glucose, fructose, ethanol, glutamate and ammonium were analyzed with kits from R-Biopharm according to the manufacturer manual, but in 1 ml total volume. Sulfate and phosphate were analyzed with “Spectroquant” kits (Merck) according to the manufacturer manual, but in 1ml total volume. As reference a standard curve was always produced.

2.3.7 Growth calculation

$$\text{Specific growth rate} \quad \mu = \frac{\ln N_1 - \ln N_0}{t_1 - t_0} \quad (2)$$

$$\text{H-value (hourly increase factor)} \quad H = e^{\mu}; t = 1 \quad (3)$$

$$\text{Numbers of fissions} \quad n = \frac{\log N_1 - \log N_0}{\log 2} \quad (4)$$

$$\text{Fission rate} \quad \nu = \frac{n}{t} \quad (5)$$

$$\text{Generation time} \quad g = \nu^{-1} \quad (6)$$

$k_{\text{DCW/OD}}$ = DCW/OD-ratio (determined through parallel measurement)

N_1 = cell count at time 1

N_0 = cell count at time 0

t = time [h]

2.4 Nucleic acid related methods

2.4.1 DNA-Isolation from *S. cerevisiae*

Purification was performed according to [Nasmyth und Reed \(1980\)](#). From a 20 ml over night culture 10ml or cells representing an optical density of 30 were resuspended in 500 μl 1 M sorbitol, 0.1 M Na-citrate, pH 7.0, 60 mM EDTA. After addition of 20 μl zymolyase 20T (5 mg ml^{-1}), 25 min shaking incubation at 37°C was performed. Prove of successful protoplasting was microscopically done. Cells were centrifuged (2 min, 3000 rpm, 4°C) and incubated in 500 μl 0.5% SDS, 100 mM Tris/HCl pH 9.7, 50 mM EDTA and fresh added RNase for 20 min at 70°C. After incubation 200 μl 5M Na-acetate was added, mixed and 45-60 min chilled on ice. Centrifugation followed for 10 min with 100% speed at 4°C. DNA from supernatant was precipitated with 700 μl 2-propanol. Pellets were washed with 70% EtOH, dried and sterile solved in 100-200 μl dH₂O.

2.4.2 RNA-Isolation from *S. cerevisiae*

Cells representing an optical density of 200 were washed with 1ml DEPC treated water (1 ml diethylpyrocarbonate in 1 l dH₂O, autoclaved) and centrifuged to pellet the cells. These were treated with fluid nitrogen and stored for further use below -70°C. Pellet was resuspended in 700 μl buffer 1 (0.5 M NaCl, 0.2 M Tris and 10 mM EDTA, pH 7 in DEPC treated dH₂O) and 20 μl β -mercaptoethanol and filled in liquid nitrogen cooled Teflon[®]-beaker. 700 μl buffer 2 (phenol, chloroform, isoamylalcohol in 25:24:1 (v/v)-ratio) and two steel balls were added to

the frozen pellet and disruption was performed by 2 min Disintegrator-S treatment. Thawed lysate was transferred to a centrifugation tube, mixed for 10 min and centrifuged for 1 h at maximal speed (RT). Supernatant was mixed for 5 min with further 700 μ l buffer 2 and centrifuged for 45 min at maximal speed (RT). Aqueous phase was precipitated with 900 μ l buffer 3 (1 M acetate, 96% (v/v) ethanol, dH₂O; 10:70:20 (v/v)-ratio) for 20 min below -70°C and centrifuged for 2 min at maximal speed at 4°C. Pellet was washed with 100 μ l 70% (v/v) ethanol and again centrifuged for 2 min at maximal speed at 4°C. Pellet was dried for 5 min (RT) and resuspended in 100 – 300 μ l DEPC treated dH₂O.

2.4.3 Nucleic acid quantification

Quality and concentration of nucleic acids were measured at $\lambda = 260$ nm in spectrophotometer (Sambrook et al., 1989). Optical density of 1 represents 50 μ g ml⁻¹ dsDNA or 40 μ g ml⁻¹ ssRNA.

2.4.4 Agarose gel electrophoresis of DNA

For separation of DNA, samples were resuspended in 1/10 volume sample buffer (60% (w/v) sucrose pH 7.0, 20 mM EDTA, 0,025% (w/v) bromphenolblue, 0,025% (w/v) xylene cyanol). Separation was performed in 0.6 - 0.8 % agarose gels in horizontal chambers with TAE (40 mM tris/acetate, pH 8.3, 2 mM EDTA, 20 mM Na-acetate) as running buffer at 25 – 140 V for 1 - 20 h. For size standards 2 μ l GeneRuler™ DNA Ladder Mix or 5 μ l to 10 μ l *Hind*III restricted λ -phage DNA were used. Gels were afterwards stained with EtBr-solution (100 μ l EtBr (1.0 mg ml⁻¹) in 500 ml TAE-buffer). Bands were visible and thus documented on the UV-transilluminator.

2.4.5 Agarose gel electrophoresis of RNA

0.75 g agarose was cooked in 36.5 ml DEPC treated dH₂O and cooled to ca. 60°C. 5 ml 10 x MEN (200 mM 10 x MOPS (0.2 M MOPS, 0.05 M Na-acetate, 0.01 EDTA, pH 7), 50 mM NaOAc, 10 mM EDTA in DEPC treated dH₂O, pH 7) and 8.25 ml 16.5% (v/v) formaldehyde was mixed and poured into the electrophoresis chamber. 7 μ l sample (ca. 30 μ g RNA) with 10 μ l FA-mix (formamid, 10 x MEN, formaldehyde; 80:18:24 % (v/v)-ratio) was denatured for 10 min at 65°C and subsequently mixed with 3 μ l loading buffer (48% (v/v) formamid, 10% (v/v) 10 x MOPS, 17% (v/v) 37% formaldehyde, 15% (v/v) DEPC-dH₂O, 7% (v/v) 80% (v/v) glycerol and 2.5% (v/v) saturated bromphenolblue) and 1 μ l 1 mg ml⁻¹ EtBr. Gel ran at 160 V for 75 - 90min.

2.4.6 Northern blot

RNA containing gel was washed for 10 min in 10 x SSC buffer (1.5 M NaCl, 0.15 M Na₃-citrate). Gel was blotted by capillary forces onto nylon membrane with 10 x SSC buffer over night and covalently linked with ultraviolet light in a UV-crosslinker.

2.5 Protein related methods

2.5.1 Cell disruption

Harvested cells were centrifuged in 200 ml aliquots at 4000 rpm for 15 min. Wet cells were weighted and resuspended in 200% (w/w) cell disruption buffer (1 x PBS (150 mM NaCl, 8.4 mM Na₂HPO₄, 1.6 mM NaH₂PO₄, pH 7.4), 0.5% (v/v) Triton X100, 1 mM PMSF). Mixture was disrupted with glass beads, 0.75 – 1.0 mm in diameter (Roth, A554.1), for 30 min in Dynomill continuously or for 15 min in Biomatik-Celldesintegrator-S in batch mode. Both devices were cooled with cool water or ice during process.

2.5.2 Determination of total protein (Bradford)

Tests were performed with Bio-Rad protein detection kit according to the manufacturer manual. A calibration curve was produced and compared with the results of the samples.

2.5.3 Immunofluorescence

Yeast cells were grown until early exponential phase and fixed through addition of 0.5 ml 37% formaldehyde to 5 ml cell culture and incubated on shaker for 10 min at 28°C. Cells were centrifuged for 5 min at 5000 rpm and resuspended in 5 ml buffer 1 (40 mM KPO₄ (pH 6.5), 0.5 mM MgCl₂) and 0.5 ml 37% formaldehyde and incubated for 1 h at 28°C. Cells were washed twice in buffer 1 and once in buffer 2 (buffer 1 with 1.2 M sorbitol). After that cells were resuspended in 0.5 ml buffer 2. Protoplasts were produced through addition of 30 µl zymolyase-solution (10 mg ml⁻¹ zymolyase 20T) and incubation at 28°C for 1 h. Progress was microscopically examined. Formation of protoplasts was stopped through washing in sorbitol buffer with gentle centrifugation at 3000 rpm. Teflon masked immunodetection glass slides with 8 wells were treated each well with 10 µl 0.1% polylysine-solution and incubated for 30 sec. Polylysine was removed by pipette and wells were washed 5 times with dH₂O. Dried wells were incubated with 20 µl of protoplasts for 1 min. Wet wells were incubated in blocking-solution (PBS+BSA) for 10 min at RT. 10 µl antibody-solution (1 x PBS (40 mM K₂HPO₄/KH₂PO₄ pH 6.5; 0.5mM Mg Cl₂), 1 mg ml⁻¹ BSA, 1:500 diluted LMW-antibodies) were added to each well and incubated in humidified chamber for 1 h. Three 15 min washing steps with blocking-solution were performed before 10 µl second antibody-solution (PBS+BSA+1:500 diluted FITC-labeled rabbit anti-mouse antibody) was added and again incubated for 1 h. Three washing steps were repeated and completely removed before mounting-solution (1 g l⁻¹ p-phenylenediamine, 2.25 mg l⁻¹ DAPI-solution, 0.1 x PBS pH 9.0

and 90% (v/v) glycerol) was added to wells and coverslip was bubble-free put on and sealed with clear nail polish.

2.5.4 Determination of specific protein amount

Because of low amounts of recombinant proteins built up from yeast, the quantification is semi quantitative. HMW-subunits can be visualized by western blot when they are also visible in coomassie stained SDS-PAGE, because of the high detection limit. First antibody for LMW6-subunit was of lower detection limit. Thus quantification of HMW-subunits was achieved after organic extraction in coomassie stained gels, whereas LMW6-subunit could be detected in complex mixtures and from extractions. Quantitative comparison was performed with Kodak image station 440 CF and the delivered software “Kodak 1D Image Analysis” for band quantification.

2.5.5 Protein extraction

LMW-subunits

Yeast cells representing an OD of 2.5 (fresh, from over night cultures or frozen) were centrifuged (6000 rpm, 5 min, 4°C). Pellets were resuspended in 200 µl 0.1 M NaOH and centrifuged again. Pellet was cooked for 3 min in sample buffer (62.5 mM tris-HCl pH 6.8; 25% (v/v) glycerol; 2% (w/v) SDS; 0.01% (w/v) bromphenolblue; 2.5% (w/v) DTT). Mixture was centrifuged again to avoid loss by condensation. Supernatant removed and homogenized by mixing. Up to 20 µl of supernatant was used for SDS-PAGE.

HMW-subunits

Yeast cells representing a OD of 50 (fresh, from over night cultures or frozen) were transferred into a 2 ml reaction tube with screwing cap and 500 µl glass beads and 300 µl disruption buffer (10 mM Na₂HPO₄ x 7 H₂O, 150 mM NaCl, 5 µl ml⁻¹ Triton X100, 10 µl ml⁻¹ 0.1 M PMSF). Cell disruption was performed in “Thermo Savant FastPräp” three times (30 s, level 6) with cooling steps for 2 min on ice. Disruption was controlled microscopically. Lysate was transferred into a 1.5 ml tube, glass beads were washed with 300 µl disruption buffer that was added to the lysate. Proteins were precipitated by addition of 600 µl 2-propanol and centrifugation (9000 rpm, 20 min and 4°C). Pellet was resuspended in 500 µl extraction buffer (50% 2-propanol, 50% disruption buffer, 1% (w/v) DTT). Incubation for one hour at 60°C and occasionally mixed. After centrifugation (14000 rpm, 20 min, 40°C) supernatant was stored at -20°C or mixed with SDS-sample buffer (100% (v/v), with 60% glycerol). 10 to 20 µl were put on the gel.

2.5.6 Sodiumdodecylsulfate-polyacrylamid-gel electrophoresis (SDS-PAGE)

Separation was performed under denaturing conditions as recommended in Mini Protean 3 Cell Instruction Manual (Bio-Rad) according to Laemmli (1970). Separation occurs at 200 V for 50 to 60min in running buffer (25 mM Tris, 191.8 mM glycine, 1% SDS (w/v), pH 8.3). Size standard was 5 μ l “Protein Molecular Weight Marker” (MBI) or purified mixtures of WSP. Gels were stained with coomassie-staining solution (0.25% Coomassie Brilliant Blue, 50% Methanol and 10% Acetate).

2.5.7 Western blot to nitrocellulose and PVDF-membranes

LMW-subunit

Transfer was performed to nitrocellulose membrane with transfer buffer (25 mM tris, 192 mM glycine, 10% Methanol, pH \approx 8.4) in a semi-dry blotter with 1 mA cm⁻² gel area for ca. 2 h (or at 50 mA over night at 4°C). In order to control the blotting membrane was stained with ponceau S (5 g l⁻¹ ponceau S, 1% acetate) and the gel with coomassie-staining solution.

HMW-subunit

Discontinuously transfer to PVDF-membrane was performed according to a protocol of the manufacturer (Serva). Three whatman papers in gel size were soaked in anode-buffer I (300 mM tris, 20% (v/v) methanol, pH 10.4) and placed onto anode. Three in anode-buffer II (30 mM tris, 20% (v/v) methanol, pH 10.4) soaked papers were stack again on anode. PVDF membrane pretreated according to manufacturer was placed on the papers and gel was placed above. Three papers soaked in cathode-buffer (25 mM tris/HCl (pH 9.4), 40 mM 6-aminocaproic acid, 20% (v/v) methanol) were placed on top. Transfer was performed at 1mA cm⁻² gel area for ca. three hours (or at 50 mA over night at 4°C).

2.5.8 Antibody-labeling of membrane bound proteins

Whole treatment was performed at RT and all washing steps for 10 min. Membranes were washed twice in TBS-buffer (150 mM NaCl, 10 mM Tris/HCl pH7.5). Blocking for 1 h in blocking-solution I (30 g l⁻¹ BSA, 10 mM tris/HCl pH 7.5, 150 mM NaCl) with following two washing steps with TBS-T buffer (0.5 M NaCl, 40 mM tris/HCl pH 7.5, 0.2% Triton X100, 0.05% Tween 20) and once in TBS-buffer were performed. Hybridization in blocking solution I with LMW6 or HMW antibodies (1:1000 diluted) and again two washing steps with TBS-T buffer and one in TBS-buffer were done. After that blocking-solution II (5 g l⁻¹ milk powder, 75 mM NaCl, and 5 mM tris/HCl pH 7.5) with POD-conjugated rabbit-anti-mouse-antibodies

(1:5000 diluted) was hybridized. Membrane was finally washed four times with TBS-T buffer.

2.5.9 Antibody detection

Membranes from western, southern or northern blot were treated according to the manual of Pierce and documented with Kodak image station with exposures from 2 to 60 min. Eventually an exposure of roentgen films were done.

2.6 Lipid related methods

2.6.1 Lipid extraction

A sample of 0.4 g were mixed with 1.5 ml methanol/chloroform (2:1 (v/v)) and incubated for one hour (RT). Mixture was centrifuged 10 min at 10000 rpm (RT) and supernatant was incubated with 0.5 ml dH₂O and 0.5 ml chloroform for 30 min and mixed several times. Mixture was centrifuged for 4 min at 5000 rpm (RT) for completion of phase separation. Lower phase was isolated and dried by applying nitrogen and warming in 40°C water bath. Dried lipid extract was resuspended in 100 µl methanol/chloroform-mixture (1:1(v/v))

2.6.2 Lipid thin layer chromatography (TLC)

Chromatography chamber is humidified with the help of filter paper and 150 ml running mixture (petrol ether, diethyl ether and acetate in a 90:10:1 (v/v) ratio). Lipid extracts were put on a HPTLC-silica gel 60 plates (Merck, Darmstadt) with 2.5 cm distance from each other and 2 cm from the border of plate. Run was started by inserting the plate into the chamber and ended after ca. two hours.

2.6.3 Iodine staining of lipids

Dried chromatography plate was incubated in an iodine saturated chamber for 10 min. Lipids turn brown at day light and black under ultraviolet light. Plates were documented by photographing.

2.7 Aqueous two-phase related methods

2.7.1 Determination of binodal of aqueous two-phase systems

The determination was performed with the so called “cloud point method” ([Hatti-Kaul, 2000](#)). A defined weight of a system component stock solution (S1) was prepared. Drop-wise addition of the second system component stock solution (S2) results after intensive mixing to slight turbidity (cloud point). The mixture was weighted and a defined amount of S1 was

added. The mixed and weighted tube appears clear and drop-wise addition was again followed.

The noted weights of the cloud points can be used to calculate the concentrations in the mixture and thus the binodal.

2.7.2 Aqueous two-phase systems calculation

$$\text{Partition coefficient} \quad K = \frac{c_t}{c_b} \quad (8)$$

$$\text{Volume ratio} \quad V_r = \frac{V_t}{V_b} \quad (9)$$

$$\text{Mass balance} \quad G = K \times V_r \quad (10)$$

$$\text{Yield} \quad Y = \frac{100}{1 + V_r \times K^{-1}} \quad (11)$$

c_t = concentration of soluble in top phase

c_b = concentration of soluble in bottom phase

V_t = volume of top phase

V_b = volume of bottom phase

3 Results

The improvement of yield and productivity is the basis of the contemporary industrial production of chemical and biotechnological products. For the increase of the efficiency of a biotechnological process it is necessary to illuminate individual steps and main factors of costs. A biotechnological production process is divided in upstream and downstream processing. Upstream processing is the cultivation of the production strain from the starter culture up to the necessary biomass amount and downstream processing is the purification of the desired product. Both fields are very facet-rich and highly different between different products.

The following chapters are divided in two parts. The first part presents the results for the upstream processing, particularly media and process optimization. Different compounds were tested for the cultivation of the selected strains. This knowledge was transferred to higher cultivation volumes and further parameters were analyzed. The second part deals with the downstream processing and thus with the recovery of the product from the yeast cells. Two approaches were investigated. Firstly the adaptation of the classical Osborne-fractionation and secondly an alternative food compatible aqueous two-phase extraction system was established.

3.1 Optimization of upstream processing

3.1.1 Shake flask cultures

In parallel to the optimization of the process, the production strain AH22 and the expression plasmids were further developed by [Hinzmann et al.](#) Due to the observation of partial secretion and posttranslational modifications of synthesized proteins, genomic deletions were identified that improved the properties of the strains. Deletion of mannosyl transferase ($\Delta pmt1$) led to a reduced O-glycosylation accompanied by a strongly reduced secretion. Final glycosylation step commonly takes place during secretion of synthesized protein. Deletion of protease B ($\Delta prb1$) reduced degradation that was observed as multiple bands in western blots. Adaptation of codon-usage and the shortening of the signal sequence of the expressed genes led to a further increase of the protein production. The reduction of the episomal plasmid size through removal of the bacterial part ($\Delta Pst1$) and partial deletion of the *ura3* promoter (*ura3d*) improved the expression of the target gene through two factors. On the one hand the plasmid size was smaller and therefore faster replicated. On the other hand the partial deletion of the

ura3 promoter caused a decrease of the expression of uracil, which only could be complemented through an increase of the plasmid copy number.

In order to verify the different plasmid constructs and genomic deletions, comparisons of the strains were performed under production condition. Firstly, the growth of the two strains AH22*ura3* YpADH-LMW6 and AH22*ura3* YpADH-LMW6 Δ *Pst1* was compared in DS-medium in shake flasks. The plasmid size reduction (Δ *Pst1*) resulted in a faster growth after or during the diauxic shift. Comparison of AH22*ura3* YpADH-LMW6 and AH22*ura3* Δ *pmt1* YpADH-LMW6 showed no difference in growth, but intracellular protein concentration showed higher concentration with increased stability. The deletion of an important protease (Δ *pep4*) led to reduced growth and to 70% lower maximal optical density compared to the parent strain. This strain was not used for further experiments.

Final strain for the investigation of LMW6 production was the resulting strain of former mentioned manipulations: AH22 *MATa*, *leu2-3*, *leu2-112* *his4-519*, *can*, *ura3*, Δ *pmt1* Δ *prb1* YpADH URA3 LEU2d LMW6.

3.1.1.1 Medium optimization

The cultivation of microorganisms presents low requirements for medium components. Microorganisms are able to form complex compounds and constituents from relatively few basic materials (essential nutrients). Most important nutrient is the carbon source. It is indispensable for biomass formation and energy generation. Additionally the yeast requires nitrogen, phosphorus, potassium, sulfur, magnesium and several trace elements. It is important that all nutrients are provided in suitable, assimilable forms.

S. cerevisiae shows defects in a few enzyme systems for the synthesis of vitamins. Therefore, the yeast strains require biotin, pantothenic acid and myo-inositol as medium supplements. In some cases also pyridoxine and thiamine are required.

Synthetic media were usually chosen to investigate the influence of individual media components on yeast cell growth and product formation (e.g. heterologous proteins). The already existent DS-medium (Hinzmann, unpublished results) was used as starting point for the optimization of the medium, which should result in high growth rate and high production of heterologous expressed wheat storage proteins.

3.1.1.2 Influence of the carbon-source

Suitable C-sources for yeasts are a great number of water-soluble, oxidized hydrocarbons, as alcohols, ketones, esters, acids and aldehydes. Commonly mono-, di- or trisaccharides are

used. The choice of C-source is also dependent on the completeness of metabolization. Numerous C-sources are not or only partially converted, whereas glucose, fructose, mannose, galactose, maltose, sucrose and turanose were completely converted by *S. cerevisiae* (Yoon et al., 2003).

Although almost all of the listed C-sources run into the glycolysis, the influence of every individual C-source on product formation and growth is not foreseeable. The uptake and metabolization are, due to the importance for the organism, strongly regulated. Acetate, ethanol, fructose, galactose, glucose, glycerol, maltose, sorbitol and sucrose were selected for the investigation of growth and product formation in shake flasks.

Initial concentration of carbon source was defined by testing. Low concentration resulted in low, hardly comparable values of optical density. Therefore, shake flask cultivations with strain AH22ura3 *Δpmt1* YpADH LMW6 in DS-media with 50, 75 and 100 g l⁻¹ sucrose were carried out to find a carbon concentration range suitable for further experiments. Maximal optical density of 53, 23 and 21 were reached after 80 h of cultivation, respectively. In the ethanol production phase the measured pH decreased to 4.7, 4.4 and 4.1, respectively. Only in 50 g l⁻¹ sucrose containing medium the pH subsequently increased after the ethanol formation phase, whereas both higher sucrose concentrations led to constant pH during the later growth phase. 18.9, 35.2 and 52.3 g l⁻¹ ethanol were produced, respectively.

Therefore, in further experiments the strain Mü4WSP was cultivated in buffered medium with carbon content equivalent to 50 g l⁻¹ sucrose. Samples were taken after 72 h and analyzed for biomass (optical density) and recombinant protein formation (coomassie stained gels for HMW-subunits and western blot detection for LMW6 subunit). First cultivations were carried out at pH 7 buffered with 150 mM potassium phosphate, due to avoids strong drop of pH. Ammonium sulfate was added as sole nitrogen source in order to maintain selection pressure and exclude the influence of complex nitrogen sources. Optical density and protein levels were shown in Table 1 and normalized to sucrose containing culture.

Unfortunately, the highest optical density reached in this experiment was 16, achieved by sucrose containing medium. Although growth was obviously limited, first indications were given by recombinant protein quantification. Interestingly, the amounts of all WSPs were reduced in glucose and fructose in comparison to sucrose containing medium. Galactose, ethanol, glycerol, acetate and sorbitol showed further reduced growth, so that WSP amount could not be determined. Despite the poor growth, galactose, maltose and ethanol containing cultures produced similar specific LMW amounts compared to sucrose containing cultures.

Table 1: *S. cerevisiae*: Influence of different C-sources on the growth and WSP formation in shake flasks
Optical density (OD) of *S. cerevisiae* (Mü4WSP) and the specific WSP production after 72 h were investigated; values were normalized to values of culture with sucrose as sole carbon source

Carbon source	OD	LMW6 yield		HMW-1Dx5 yield		HMW-1Dy10 yield	
		specific	total	specific	total	specific	total
Sucrose	100%	100%	100%	100%	100%	100%	100%
Glucose	100%	58%	58%	28%	28%	27%	27%
Fructose	91%	80%	73%	84%	76%	83%	76%
Galactose	3%	95%	3%	*	*	*	*
Maltose	3%	84%	3%	*	*	*	*
Ethanol	7%	102%	7%	60%	4%	64%	4%
Glycerol	15%	58%	9%	100%	15%	89%	13%
Acetate	2%	*	*	*	*	*	*
Sorbitol	2%	*	*	*	*	*	*

* samples could not be taken because of low biomass formation

Total amount of recombinant protein was obviously reduced. Detected amounts of HMW subunits showed similar production rates for both 1Dx5 and 1Dy10 subunits. Interestingly, glucose containing media showed lower HMW amounts than fructose containing medium. Glycerol promoted HMW production compared to LMW, whereas protein production in ethanol behaved vice versa.

Because of the incomplete conversion of the C-sources, a limitation of other medium components was supposed. In order to exclude nitrogen as a factor, complex nitrogen sources were added to the medium. 20 g l⁻¹ Na-glutamate were added to the shake flasks in the first set, 20 g l⁻¹ Na-glutamate and 2.5 g l⁻¹ NZ in the second set. 2xDS-medium was used as basis, but buffered at pH 5.5. Biomass and protein amount were determined as mentioned above (Table 2).

Maximal achievable biomass for sucrose containing cultures increased with the addition of complex nitrogen by the factor of 2.7. The growth on glucose, fructose, maltose, acetate and sorbitol were not affected relatively to sucrose. Growth on the non-fermentable carbon sources ethanol and glycerol benefited from the complex nitrogen and reached higher maximal optical densities. For ethanol containing cultures an increase of the relative optical density from 7% to 89% could be reached. Similar results were obtained with glycerol containing cultures with an increase of the relative optical density from 15 to 63%. The total LMW amount was not significantly affected by the addition of a complex nitrogen source for most of the tested carbon sources.

Table 2: *S. cerevisiae*: Influence of DS-medium with diff. C-sources and glutamate (glu) or NZ-amines (+NZ) on growth and WSP formation in shake flasks
Optical density (OD) of *S. cerevisiae* (Mü4WSP) and the specific WSP production after 72 h, values were normalized to values of culture with sucrose as sole carbon source

Carbon source	Glu OD	+ NZ	+NZ		+NZ		+NZ	
			LMW6 yield specific	total	HMW-1Dx5 yield specific	total	HMW-1Dy10 yield specific	Total
Sucrose	100%	100%	100%	100%	100%	100%	100%	100%
Glucose	96%	98%	60%	59%	87%	85%	84%	82%
Fructose	98%	102%	69%	70%	81%	83%	77%	79%
Galactose	5%	6%	87%	5%	*	*	*	*
Maltose	3%	4%	93%	4%	*	*	*	*
Ethanol	45%	89%	142%	126%	82%	73%	70%	62%
Glycerol	42%	63%	128%	81%	190%	120%	171%	108%
Acetate	1%	5%	110%	6%	*	*	*	*
Sorbitol	2%	2%	145%	3%	*	*	*	*

* samples could not be taken because of low biomass formation

Only ethanol and glycerol contributed to high specific and total LMW-amounts, resulted from improved growth. For ethanol this value was even higher than for the reference cultures. HMW formation also benefited from the complex nitrogen. Especially glucose containing cultures reached values similar to that of fructose. Ethanol containing cultures showed a slight and glycerol a strong increase in relative specific HMW-amount. The total amount was obviously increased by the improved growth. For glycerol the detected HMW amount exceeded that of sucrose containing cultures. Despite the high specific product formation in glycerol containing cultures, sucrose represented the best carbon source for the given cultivation conditions.

3.1.1.3 Influence of the N-source

The N-sources presented in DS-medium are a mixture of ammonium, glutamate and a protein hydrolyzate (CA). All three represent well relevant N-sources, whereby glutamate and CA through their available carbon scaffold provide also a relevant C-source.

AH22 $ura3\Delta pmt1$ YpADH LMW6 was cultivated in DS-medium varied in nitrogen sources as shown in Table 3. Na-glutamate was provided to avoid pH drop, ammonium sulfate and ammonium phosphate to supply sulfur and phosphor. 0.3 g l⁻¹ leucine was supplemented to complement the leucine auxotrophy of the used strain. Plasmid maintenance was not disturbed by this, because plasmid bearing cells were selected by uracil auxotrophy. Samples were taken after 48 and 72 h, optical density and the amount of LMW were determined by western blot. Two different sources of protein hydrolyzates (NZ and CA) were tested in different amounts, in order to investigate the influence of different amino acid compositions.

Ammonium, glutamate and urea were tested as alternative cheap nitrogen sources. High amounts of amino acids arginine and asparagine were also investigated, because of the positive influence to product formation reported by Gorgens et al. (2005) and an inhibitory effect to protease activities described by Gaczynska et al. (2003).

Table 3: *S. cerevisiae*: Influence of DS-medium with diff. N-sources on growth and LMW6 formation in shake flasks
Optical density (OD), specific and total (calculated by multiplication of spec. LMW6 with optical density) LMW production after 48 and 72 h; values were normalized to values of DS-medium

Medium	OD		specific LMW6-yield		total LMW6-yield	
	48h	72h	48h	72h	48h	72h
DS (5gl⁻¹ CA)	100%	100%	100%	100%	100%	100%
DS with 5gl ⁻¹ NZ	95%	105%	98%	104%	93%	109%
DS with 10gl ⁻¹ NZ	101%	108%	111%	high	112%	high
DS with add. 5gl ⁻¹ NH ₄ SO ₄ in substitution to CA	81%	86%	110%	128%	89%	110%
DS with add. 10gl ⁻¹ Na-Glu	103%	109%	98%	120%	101%	131%
DS with 2gl ⁻¹ urea	87%	88%	96%	88%	84%	77%
DS with add. 2gl ⁻¹ Leu	92%	96%	53%	67%	49%	64%
DS with add. 0.3M Arg	82%	76%	109%	high	89%	high
DS with add. 0.3M Asp	62%	69%	130%	high	80%	high
DS with add. 1mM PMSF	64%	59%	83%	high	53%	high

high – much higher values than reference because of extended growth

Cultures containing NZ instead of CA reached slightly higher optical density, LMW-amount and extended protein stability. Further increase of the NZ concentration also increased growth and product formation, but in relation to the high amount of supplementation this effect is only marginal and expensive. The complete lack of CA by substitution through ammonium sulfate resulted according to section “influence of the carbon source” in reduced growth but a higher amount of product could be observed after 72 h. The increase of Na-glutamate concentration led to an improved growth and to a significant increase of specific and total product formation. The measurement of glutamate consumption in the culture supernatant revealed a consumption of 12 g l⁻¹ for the assimilation of 50 g l⁻¹ sucrose in the 20 g l⁻¹ Na-glutamate containing culture.

The addition of 2 g l⁻¹ urea to the medium resulted in a prolonged lag-phase of 60 to 140 h and to decreased growth and WSP formation. Also the increase of the leucine concentration to 2 g l⁻¹ in DS-medium led not to an improvement. This shows sufficient concentration of leucine in the reference cultivation.

Although high amounts of arginine and asparagine result in slower growth, an increased relative specific LMW6-amount for an extended time after reaching stationary phase up to

140th hour could be achieved. Comparison to protease inhibitory PMSF showed similar reduced growth rate and prolonged intracellular protein content.

Nitrogen provision in DS-medium was improved by an increase of total nitrogen. Especially glutamate as the central nitrogen source for yeast and NZ led to improved growth and product formation.

3.1.1.4 Influence of mineral salts

Mineral salts are important growth factors for the yeast *S. cerevisiae*. Certain minerals are of particular interest, whereas others as sodium, silicon, calcium and chlorine are relatively less important (Bronn, 1986). Minerals often represent cofactors for or main components of proteins and enzymes. To exclude limitations in DS-medium, different concentrations of all mineral components were tested. 0.5x, 2x, 4x and 8x-DS-medium were tested for growth. Only the carbon content was kept constant. 4x and 8x-DS-medium showed inhibitory growth effects, whereas 0.5x-DS-medium showed limitations, which were shown through incomplete carbon metabolization and a decreased final optical density. 2x mineral concentration were found to be best for growth and were therefore used for subsequent investigations.

To further investigate the influence of single mineral components all mineral medium components were varied in shake flask experiments. 2xDS-medium was buffered with potassium phosphate buffer at pH 7. Maximal attainable optical density was ca. 50 on 2xDS-medium with 50 g l⁻¹ sucrose and ammonium as sole N-source. Ammonium concentration was increased to compensate nitrogen loss through the lack of complex nitrogen sources. Variation was performed in three steps.

Table 4: *S. cerevisiae*: Influence of diff. components (except 5% sucrose) on growth in shake flasks
Optical density (OD) was investigated after 258 h, values were normalized to maximal values, and media were based on 2x-DS-medium (for further information see text)

compound-no.	conc. [g l ⁻¹]	% in 2xDS	OD	compound-no.	conc. [g l ⁻¹]	% in 2xDS	OD
NH ₄ -1	0,435	7%	59%	Cl ₂ -1	0,074	7%	90%
NH ₄ -2	1,696	27%	88%	Cl ₂ -2	0,673	33%	91%
NH ₄ -3	3,396	54%	87%	Cl ₂ -3	1,610	66%	89%
SO ₄ -1	0,429	2%	57%	Ca-1	0,015	18%	92%
SO ₄ -2	1,610	9%	68%	Ca-2	0,055	55%	90%
SO ₄ -3	3,366	19%	87%	Ca-3	0,129	92%	89%
PO ₄ -1	9,497	50%	14%	Na-1	0,002	*	95%
PO ₄ -2	18,994	100%	99%	Na-2	0,366	*	92%
PO ₄ -3	37,98	200%	85%	Na-3	0,850	*	87%
Mg-1	0,101	34%	87%				
Mg-2	0,125	51%	84%				
Mg-3	0,171	68%	89%				

* not present in DS-medium

Variation of secondary ions was balanced by an increase of other salts containing the affected ion. Concentration, percentaged part of 2xDS-medium and achieved optical density after 258 h is presented in Table 4

The results showed that 2x-DS-medium was sufficient equipped with mineral materials to metabolize 50 g l⁻¹ sucrose. Moreover, for some minerals as Mg, Ca, K and SO₄ an unnecessary high concentration in 2xDS-medium was observed. Costs for medium preparation could be saved, if results were considered for fermentations. The variation of sodium and chlorine showed no improvements of growth and led to lower optical density at higher concentrations. Different sulfate concentrations did not only influence the maximal achievable biomass, but also the specific growth rate. The growth rate was lower for lower substrate provision. Similar observation was made for phosphate at concentrations higher than in reference cultivation. Lower concentration led to drastic decreased optical density, which can also be explained by pH drop due to lower buffer capacity. In order to show importance of potassium, shake flasks experiments were performed completely lacking potassium. Highest optical density was decreased to 10% to that of potassium containing cultures.

3.1.1.5 Influence of vitamins

Shake flasks cultivations were carried out, in which the composition of vitamins was varied (Table 5). Mü4WSP was cultivated in a medium based on 2xDS-medium with 5 % sucrose and ammonium sulfate as sole N-source. Biotin, pantothenic acid, pyridoxine, thiamine and nicotinic acid were set to 20 mg l⁻¹ and myo-inositol to 250 mg l⁻¹. Biomass formation was determined by measuring optical density. Specific recombinant protein was determined by western blot and coomassie stained gels. All determined values were normalized to the reference cultivation provided with all tested vitamins.

Table 5: *S. cerevisiae*: Influence of DS-medium with diff. vitamins on growth and WSP formations in shake flasks

Optical density (OD) and the specific WSP production were investigated after 72 h; values were normalized to values of DS-medium full supplemented

Vitamin	max. OD	LMW6	HMW-1Dx5	HMW-1Dy10
With all vitamins	100%	100%	100%	100%
Without all vitamins	27%	66%	16%	14%
Without inositol ^{a)}	94%	115%	101%	100%
Without biotin ^{a)}	101%	118%	85%	89%
Without pantothenic acid ^{a)}	97%	116%	86%	90%
Without pyridoxine	100%	97%	98%	96%
Without thiamine	95%	110%	85%	89%
Without nicotinic acid	109%	100%	91%	66%

a) Classified as essential vitamins

The lack of all vitamins led to a strongly reduced optical density of 27%. In contrast, the removal of single vitamins has only minor consequences for growth. For nicotinic acid the results for growth were contrary to the expectation. The complete lack of nicotinic acid results in an improved growth of 109%, indicating no necessity for growth. Regarding the protein formation fundamental differences can be seen between LMW6- and HMW-subunits. LMW-subunits were produced in higher amounts at single vitamin removal compared to HMW-subunit, which was decreased for all vitamins except for myo-inositol. The complete lack of vitamins affected the HMW formation more dramatically than the LMW production.

In order to investigate the influence of further vitamins on the biomass and product formation, amino benzoic acid, folic acid and riboflavin were added to DS-medium. The addition of these vitamins positively affects specific growth rate and minimally the LMW6 formation. The addition of these vitamins was rejected because of low improvements.

Because of the negative effects on HMW-subunit production at removal of single vitamins and nearly no observable differences in biomass production, the provision of vitamins in DS-medium was found to be sufficient. Defined concentration demand could not be determined in this work, but could be investigated in future works.

3.1.1.6 Conclusion of media optimization

In spite of the highest specific product formation with glycerol as carbon source, growth and total product formation were determined to be best with sucrose containing medium. Provision with complex nitrogen sources especially higher concentrations of protein hydrolyzate (NZ) or glutamate increases specific growth rate, biomass and product formation. Higher specific protein concentration could be determined with addition of mentioned complex nitrogen sources as well as single amino acid supplementation (asparagine, arginine). Access to nitrogen especially in stationary phase decreases specific protein degradation, when carbon source is depleted. Phosphorus, sulfur and potassium are of major importance. They did not only influence maximal attainable biomass concentration, but also growth rate. Also important in traces are magnesium and the non-investigated trace elements of the trace element solution. Vitamins should be provided as in DS-medium composition. Further adaptation of medium composition should be considered, if carbon source concentration is increased or feeding strategies are applied as in the following sections.

3.1.1.7 Influence of the temperature

Growth behavior of the strain Mü4WSP was tested in shake flasks at 20, 28 and 37°C. Cultivations were carried out in 2xDS-medium with ammonium sulfate as sole nitrogen source. The pH value was maintained by potassium phosphate buffer as mentioned above. Biomass formation represented in optical density is presented in Figure 5.

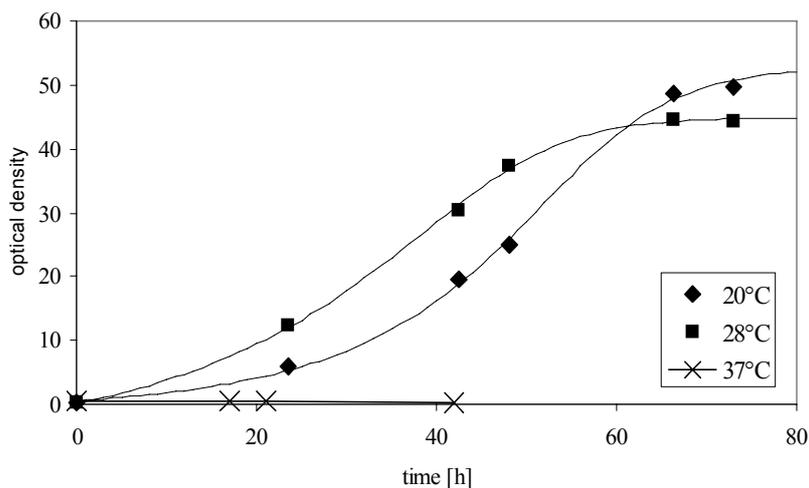


Figure 5: *S. cerevisiae* Mü4WSP: Influence of temperature (20, 28 and 37°C) on growth in shake flasks

Cultivation temperature of 37°C did not lead to any detectable growth after 42 h and cultivation was terminated. Growth arrest could indicate temperature sensitivity of the used strain. The growth at 20°C and 28°C was different mainly at the beginning of the cultivation. As expected, higher temperature led to a higher growth rate (0.14 h^{-1} , 20°C; 0.18 h^{-1} , 28°C). Whereas optical density of cultures growing at lower temperature were slightly higher at the end of cultivation.

3.1.1.8 Influence of the pH value

Strain AH22 $ura3\Delta pmt1$ YpADH LMW6 was cultivated in unbuffered DS-medium. The pH values were set to 4, 6 and 8 by addition of acid (hydrochloric acid) or base (potassium hydroxide). As shown in Figure 6A, the growth at pH 4 and pH 6 was similar. Cultivation at pH 8 resulted in a prolonged lag-phase (pH8-1) or even in no growth (pH8-2). Regarding the specific product formation of LMW6 subunit, no differences could be observed in all cases (data not shown); pH 6 led to insignificantly more protein.

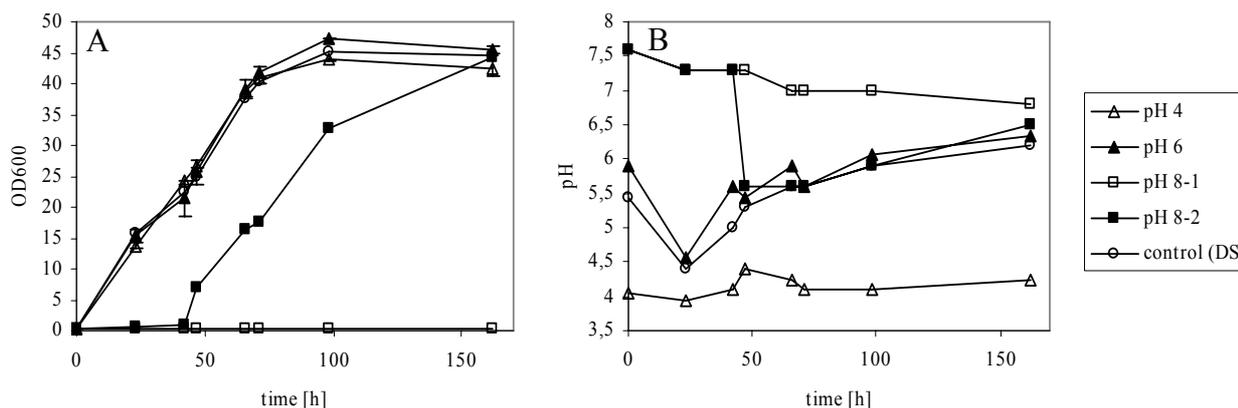


Figure 6: *S. cerevisiae*: Influence of starting pH on growth in shake flasks
 (A) optical density of shaken flasks at 600nm; (B) pH in shake flask with different starting pH values over 160h, error bars indicated duplicates, pH 4 (○), pH 6 (▲), pH 8-1 (□) -2 (■) and control (DS)

The pH values were not constant during cultivation time, but showed characteristic progression (Figure 6B). The N-source had a decisive influence on pH. Interestingly, cultivation at pH 4 did not show the expected pH progression, pH maintained close to a value of 4 during cultivation.

In the next shake flask experiments buffered DS-media supplemented with ammonium as sole nitrogen source were used in order to maintain medium pH. Media were buffered by 100 mM potassium phosphate at pH 4, 6 and 7.

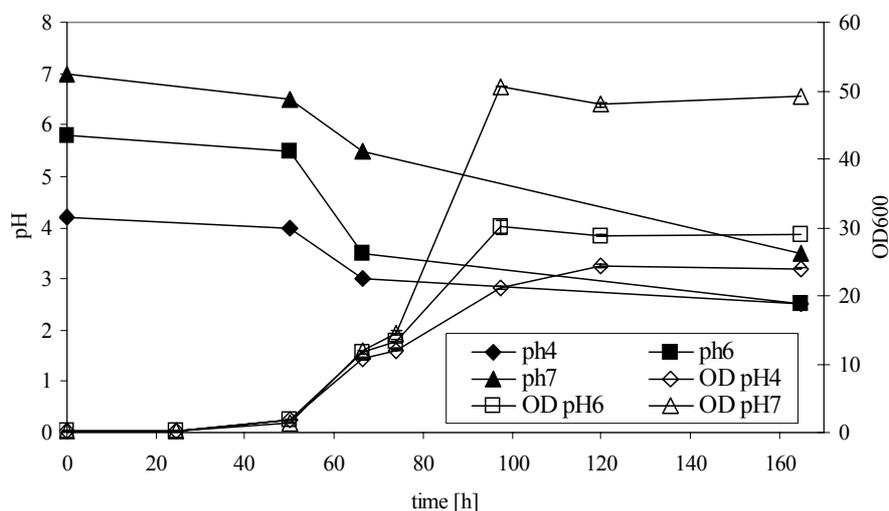


Figure 7: *S. cerevisiae*: Influence of different buffered starting pH on growth in shake flasks

Despite buffering the media with 100 mM potassium sulfate, pH decreased strongly during cultivation (Figure 7). Cultures at pH 4 and 6 reached only 40% of the maximal achievable optical density, because of a further decrease to pH 3. At the beginning growth progression

was equal for all tested pH values, but after drop of pH, achievable optical density was influenced by buffer capacity and starting pH value.

3.1.2 Batch-cultures

3.1.2.1 Influence of the pH maintenance

For the investigation of the influence of the pH maintenance two batch-fermentations, in which the pH value was controlled and respectively not controlled, were carried out. In order to avoid strong pH drop AH22ura3Δpmt1 YpADH LMW6 was cultivated in DS-medium supplemented with 12 g l⁻¹ Na-glutamate. Optical density and specific product formation was measured and is shown in Figure 8A. Western blot derived values were used to calculate total LMW6 amount visible in Figure 8B.

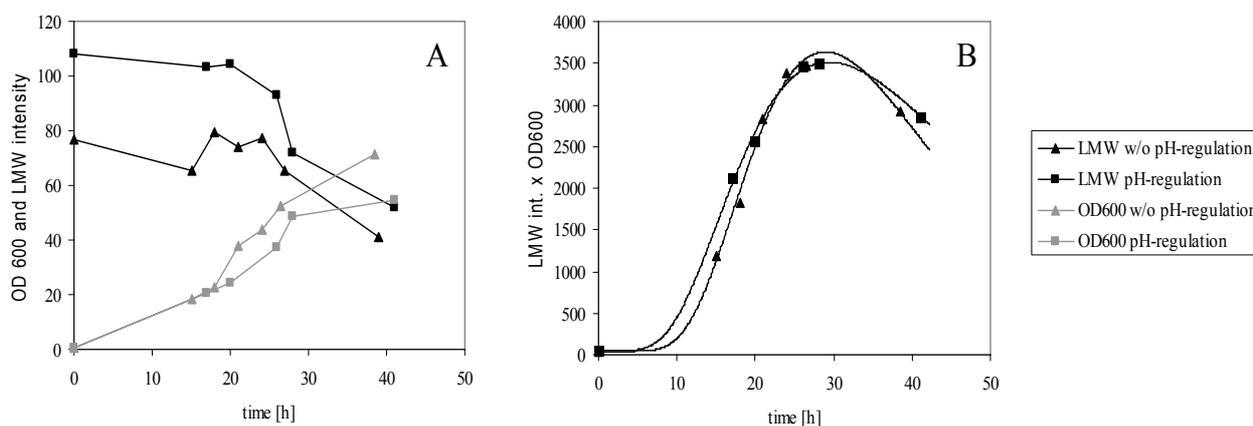


Figure 8: *S. cerevisiae*: Influence of pH-regulation on growth and WSP formation in shake flasks
(A) Optical density (grey symbols) and specific LMW6 content (black symbols), determined by intensity of western blot signals; (B) total LMW6 (calculated by multiplication of OD and specific LMW6 content), w/o pH-regulation (▲) and with regulation (■) in 2l-fermentor

Growth was similar up to 20th hour, but finally cultivation without pH-regulation reached higher optical density. Specific LMW6-amount declined during cultivation for both cultures, but cultivation with pH-regulation reached higher final value. Even if there were differences in growth and specific protein production, the total specific protein content was practically the same, as shown in Figure 8B. The addition of pH compensating components was not necessary, if glutamate was used in batch fermentation.

3.1.2.2 Influence of different regulated pH values

AH22ura3Δpmt1 pYADH LMW6 was cultivated in DS-medium at pH 3; 4.5; 6 and 7.5. Four 200 ml-fermentors were inoculated in parallel. Oxygen concentration was regulated by

stirring speed and pure oxygen supply to avoid oxygen saturation below 15%. Optical density was measured (Figure 9A) and total LMW6 formation was calculated by multiplying specific product formation (measured by western blot detection) with optical density (Figure 9B).

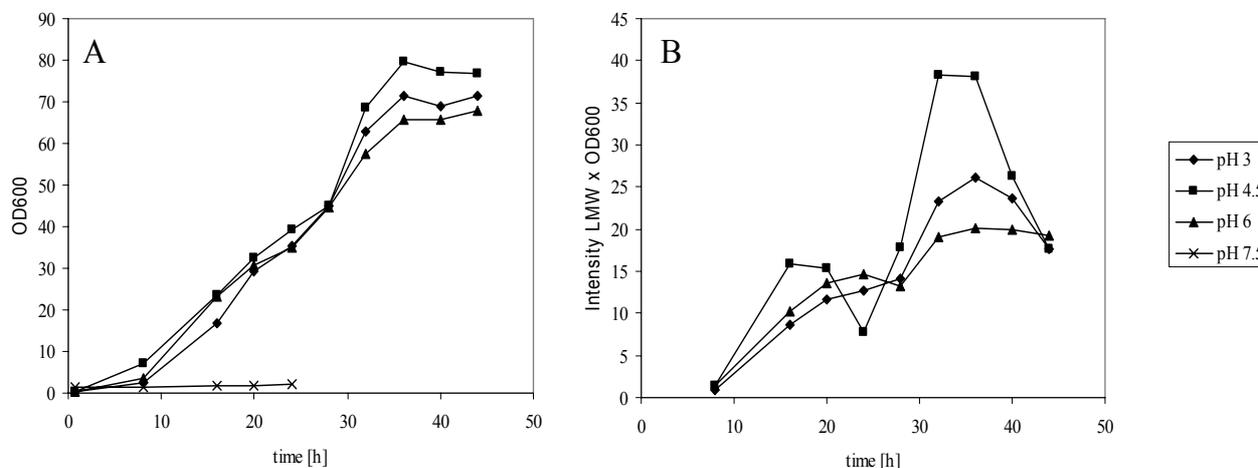


Figure 9: *S. cerevisiae*: Influence of different pH on growth and WSP formation in batch fermentation (A) optical density at pH 3 (◆) pH 4.5 (■) pH 6 (▲) and pH 7,5 (x); (B) total specific LMW6-content, calculated by multiplication of OD and LMW6 intensity measured in western blot, in 200ml fermentors

In contrast to the former results, cultivation at pH 7.5 did not lead to any detectable growth. At all other tested pH values growth behavior was comparable, with slight differences in the final biomass (pH 3 = 71; pH 4.5 = 80 and pH 6 = 66 OD after 36 h). For the specific LMW6 concentration a strong correlation between protein production and growth could be observed. As shown in Figure 9A, total LMW6 content increased up to 30th hour. Diauxic shift led to a decreased specific product formation. During the following ethanol consumption phase LMW6 formation increased again and reached its maximum before stationary phase was entered. Limitation of substrate at transition to stationary phase triggered degradation of LMW6 and resulted in decreasing total LMW6 amount at the end of cultivation. The results showed that the optimal pH value for biomass and product formation lies around pH 4.5, whereas cell harvesting should take place during the ethanol consumption phase before the culture enters the stationary phase.

Another interesting effect observed during this experiment was that the cultivation at pH 4.5, which gave the highest biomass and product yield, required the lowest oxygen amount as indicated by stirring speed (851 rpm) and oxygen gas mix (34.0%), compared to pH 3 (949 rpm, 55.2%) and pH 6 (1027 rpm, 72.6%).

3.1.3 Fed-batch-cultures

3.1.3.1 Determination of the maximal H-value

Fed-batch cultivations were performed by the feeding of highly concentrated substrates. As mentioned in the section “Theoretical Part”, feeding without feedback control is best performed with exponential feeding profile. The calculation of the volume flow is decisive for the process. The H-value represents the hourly increase factor of biomass concentration respectively optical density. This is used for the calculation of feed at the yeast “Zulaufverfahren”.

For the determination of the maximal H-value strain AH22*ura3Δpmt1* pYADH LMW6 was cultivated in DS-medium. The H-value was varied from 1.10; 1.15; 1.17; 1.19 to 1.25. Exponential feeding profile was performed through computer aided feeding. Parameters for calculation were: starting volume, substrate concentration of feeding medium (sucrose), H-value, pump factor and starting biomass concentration (calculated through optical density and estimated DCW/OD-ratio). Obtained H-value was calculated by optical density that was reached at the certain adjusted H-values (Figure 10).

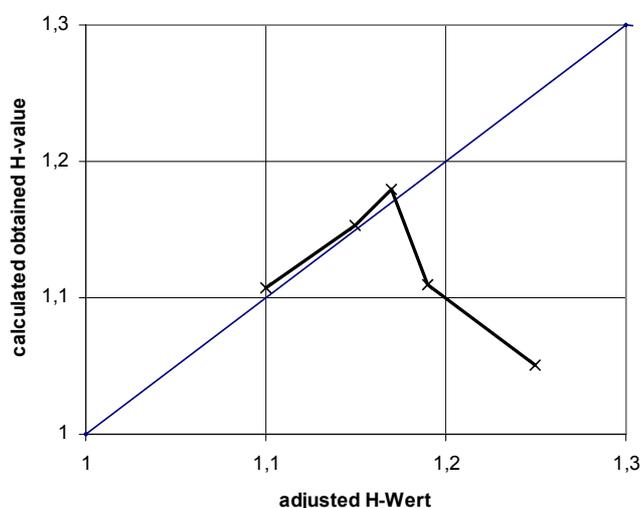


Figure 10: *S. cerevisiae*: Determination of the maximal H-value in fed-batch fermentation
On x-axis adjusted H value is shown, whereas on y-axis actual reached H-value is represented

The adjusted H-value could be obtained up to a value of 1.17. As shown in Figure 10, an increase above 1.17, which corresponds to a specific growth rate of 0.157 h^{-1} , resulted in a decrease of the actual obtainable H-value. The maximum H-value varied for the used cultivation conditions between 1.17 and 1.19. Higher values led to ethanol formation due to

exceeded Crabtree concentration. Therefore, $Y_{X/S}$ decreased and further inflow of fresh medium led to a dilution of the culture.

3.1.3.2 High cell density fermentations (HCDF)

For a maximum yield of product it is necessary to achieve very fast growth accompanied by very high biomass concentrations. Growth to high cell densities presupposes high substrate concentrations and high nutrient entries. This can not be realized in batch fermentations because high substrate concentrations, as represented in the previous chapters, are inhibitory or toxic. For this reason HCDF are realized by feeding strategies. Important parameters of achieving high cell density are the concentrations of the feed medium, the specific yield coefficients and the volumes respectively volume flows, which enters the vessel.

In batch fermentations glucose concentration considerably exceeds Crabtree-concentration. This leads to ethanol production. Only after complete consumption of the entire glucose, ethanol could be used as a C-source, due to the glucose catabolite repression. This “detour” becomes noticeable also for the decrease of the specific substrate yield coefficient. This is lowered from approx. 0.5 to 0.3. In order to lower the maximal obtained ethanol concentration and prevent growth inhibition, the initial concentration of fermentable C-sources is limited. Concentrations above Crabtree-concentration have beside the mentioned effects also advantages. They lead to specific growth rates higher in fermentative growth than in respiration.

The use of 50 g l^{-1} sucrose in the batch fermentation was accompanied by ethanol accumulation up to 25 g l^{-1} , which corresponds to a specific growth rate of 0.28 h^{-1} in the fermentative phase and thus to relatively fast growth. Ethanol development can be overcome by subsequent feed of glucose below the Crabtree-concentration. Even if ethanol is present in the medium, glucose is favored respired. This fatal disadvantage of the batch fermentation could be circumvented through substrates without catabolite repression (e.g. glycerol) or through suitable feeding strategies.

3.1.3.3 HCDF on sucrose basis (linear feeding profile)

For HCDF a yeast strain with integrative copies of HMW-1Dx5 and -1Dy10 (*AH22ura3 Δpmt1* HMW 1Dx5 1Dy10) was cultivated in 2xDS-medium. Because of strong carbon limitation, if sucrose feeding would be interrupted, 40 g l^{-1} glycerol was additionally added. Glycerol is only consumed, if sucrose is completely metabolized. In order to not exceed the maximal working volume, the volume of the culture was kept constant by continuous harvesting of fermentation broth that exceeded two liters. Despite the fact, that this cultivation

method has high similarities to continuous cultivation, steady state could not be achieved, because the feed concentration was too high. Complete metabolization would lead to a DCW of ca. 250 g l⁻¹, which, as mentioned in the section “Theoretical Part”, can not be achieved. Feed medium consisted of same components as 2xDS-medium, but supplemented with 800 g l⁻¹ sucrose and 100 g l⁻¹ Na-glutamate. The feed volume flow was low at the beginning, in order to not exceed the maximal specific growth rate and to avoid the reversion of the process to fermentative growth. After initial manual successive increase of the volume flow a constant volume flow of 0.7 ml min⁻¹ was chosen. This corresponded to slow growth at $\mu < 0.1$ (Figure 11). Due to the linear feeding profile a nearly linear increase of the dry cell substance and a logarithmic decreasing of the specific growth rate could be predicted at the beginning of the process.

Linear feeding profile, represented by the linear increase of feeding weight (Figure 11B) and constant volume, was applied to reduce oxygen demand and achieve high cell density. Despite the slower growth, maximal stirrer speed (1500 rpm) and high aeration (3.5 vvm), oxygen saturation reached zero after ca. 48 h (Figure 11B). Although oxygen limitation was observed, the process could be preceded for further 26 h by reaching an optical density of ca. 500 (Figure 11A).

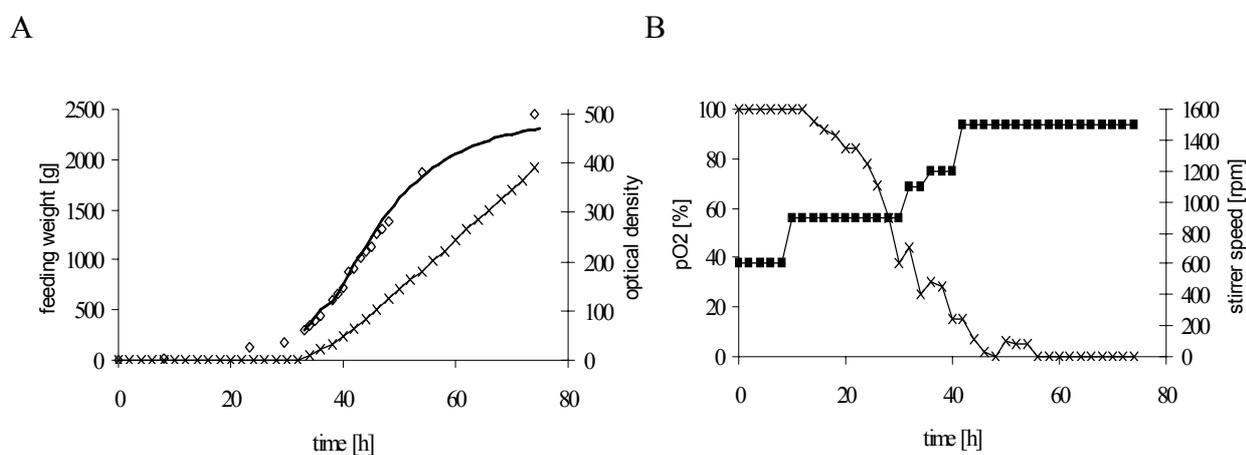


Figure 11: *S. cerevisiae*: HCDF with linear feeding profile based on sucrose as carbon source
(A) optical density (OD, \diamond); feeding weight (\times); predicted optical density (—); (B) fed-batch fermentation with linear feeding profile; stirrer speed (\blacksquare); oxygen saturation (\times)

Analysis of the supernatant (Figure 12) showed that after 48 h almost all analyzed media components were limited (sucrose, glycerol, ethanol, phosphate, glutamic acid). Only ammonium and sulfate were available in sufficient amounts, due to the pH correction with 25% NH₃ solution and 4 M H₂SO₄. Between the 54th and 60th hour the growth was more

strongly limited, through what an accumulation of the media components and the formation of ethanol occurred.

As expected after reaching a linear feeding profile a linear growth could be observed at the beginning of feeding. The productivity declined during cultivation. As shown at the progression of the predicted optical density, the maximal achieved OD was around 500. This OD represents nearly steady state of the performed continuous-like culture. Productivity was highest after 58 h and represented with $2.8 \text{ gDCW l}^{-1} \text{ h}^{-1}$ a high value, despite the long batch-phase. It slightly decreased within the last 15 h to $2.3 \text{ gDCW l}^{-1} \text{ h}^{-1}$.

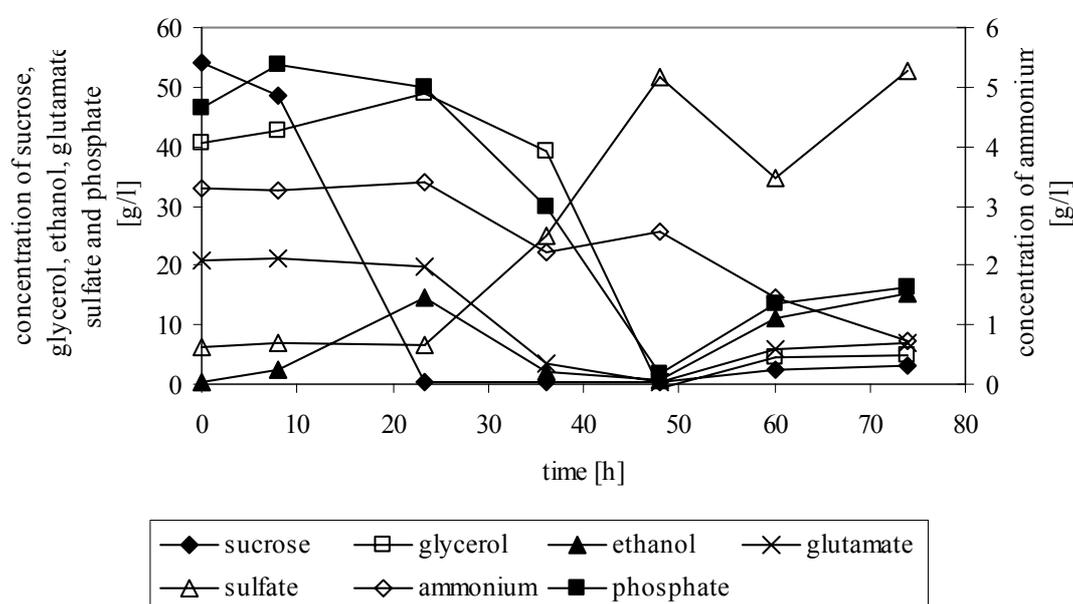


Figure 12: *S. cerevisiae*: Medium components of culture broth in HCDF with linear feeding profile
Linear feeding profile was started at 32nd hour of cultivation

Despite the long distance between sample takings, the whole process can be represented in Figure 12. Sucrose was converted to ethanol at the beginning of the cultivation (batch-fermentation). After 22 h nearly no sucrose could be measured in the medium and the ethanol concentration achieved its determined maximum. Diauxic shift is accompanied by intermittent decrease of the oxygen saturation (Figure 11). Parallel to the oxygen saturation the decrease of the concentration of the presented glycerol, ammonia and glutamate occurred. Increase of glycerol could be observed at the beginning of fermentation. At the start of feeding glycerol concentration was already decreased. The sulfate and the ammonia concentration increased meanwhile through the correction of the pH value, as already described.

Samples were taken and analyzed via quantification of coomassie stained gels for the production of HMW-1Dx5 and -1Dy10. Specific and overall HMW-subunit amount is represented in Figure 13. During the linear growth phase the specific protein formation decreased, but subsequently increased again. This was also observed at the beginning of the cultivation. At slow growth during the ethanol respiration between the 23rd and 33rd hour the specific product formation increased, whereas decrease was observed after the start of feeding up to 54th hour.

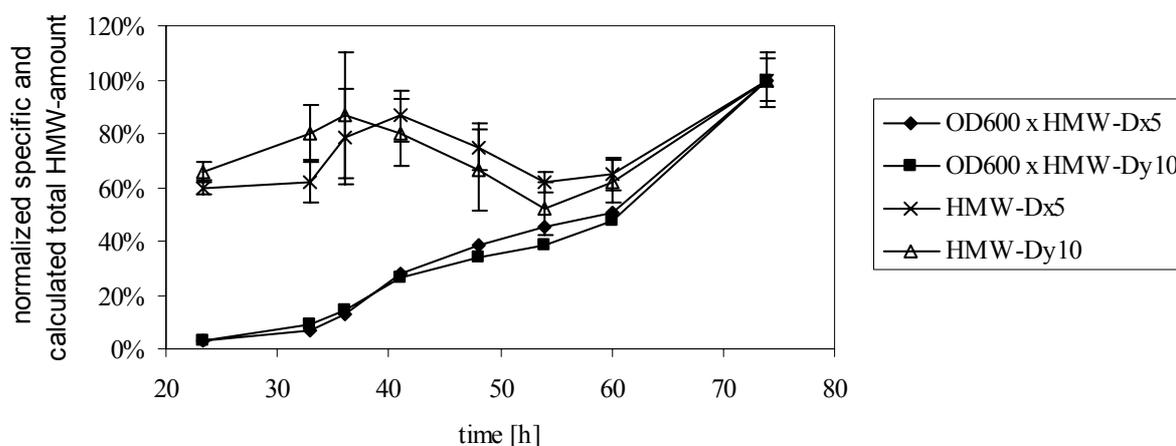


Figure 13: *S. cerevisiae*: WSP formation of HCDF with linear feeding profile

Quantification of a coomassie-stained SDS PAGE of HMW-1Dx5 (×) and HMW-1Dy10 (Δ) of fed-batch fermentation with applied linear feeding profile; total protein amount through multiplication of the specific protein amount by the measured optical density of HMW-1Dx5 (◆) and HMW-1Dy10 (■)

3.1.3.4 HCDF on sucrose basis (exponential feeding profile)

In order to avoid loss by continuous harvesting of fermentation broth, a fed-batch-fermentation was employed. The decisive parameters for high biomass yield were the start volume of the culture and the concentrations of the feed medium. Furthermore, the maximal working volume of the fermentor limited the fed-batch process. **700 ml starting volume** was chosen, which corresponds to the lowest possible volume given through the fermentor design. 700 g l⁻¹ sucrose concentration was chosen for the carbon feed. Higher values would have resulted in higher viscosity and crystallization effects, which should be severely avoided.

The start concentration in the batch phase was set to 30 g l⁻¹ sucrose, which reached an optical density of 25 after 23 h of cultivation, if inoculation led to an optical density of 0.2. Batch fermentation resulted to an Y_{xs} (fermentation) of 0.33 and to an average growth rate of 0.2 h⁻¹. Higher carbon concentrations would have led to an increased formation of ethanol and thus to slower growth. The specific growth rate continuously decreased during the batch fermentation

and increased again after the transition to the respiration. The respiration of the formed ethanol was important in two kinds. Firstly, the yield was increased again, through which the energy loss became smaller and secondly the formed ethanol would have not been consumed during feeding, because of glucose catabolite repression. High ethanol concentration would have decreased the maximum growth rate.

The complete consumption of ethanol was well recognizable by the suddenly increasing oxygen saturation, which showed reduced oxygen consumption. The subsequent start of feeding led to an immediate decrease of the percentaged oxygen saturation. An H-value of 1.17 ($\mu = 0,157 \text{ h}^{-1}$) was chosen as determined as the maximum value by previous experiments. Limitation of oxygen was excluded by oxygen supply. Oxygen saturation was set to 75% or above, when oxygen was supplied to the fermentor.

As shown in Figure 14, the predicted biomass formation and the feeding profile, represented by the culture volume, were presented. If the fermentor volume and growth would not be limited, 700 g l^{-1} sucrose and a yield coefficient of 0.5 would lead to 350 gDCW l^{-1} . Obviously, this is not possible, as described in the section “Theoretical Part”. The limited volume of the feed medium, however, led to a termination of the cultivation at the predicted dry cell weight concentration of 225 g l^{-1} .

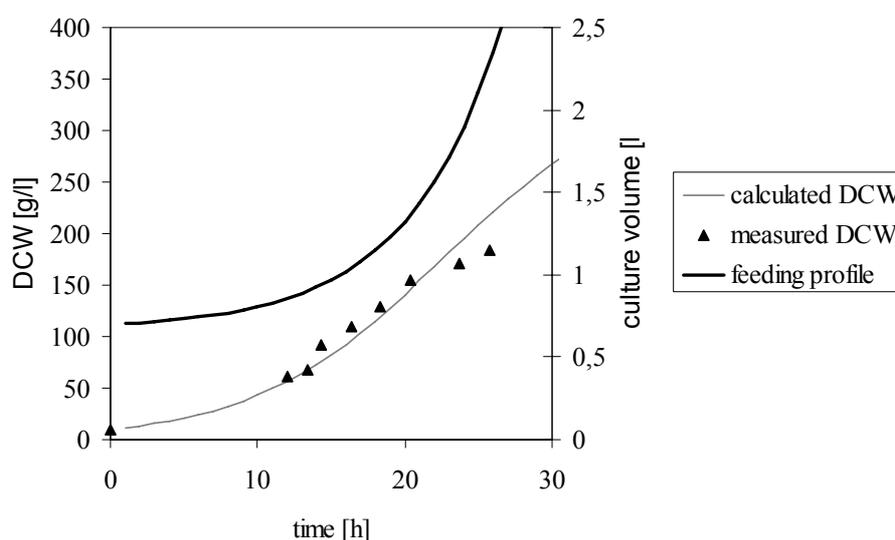


Figure 14: *S. cerevisiae*: HCDF with exponential feeding profile based on sucrose as carbon source
Exponential feeding profile (black line), predicted dry cell weight concentration (grey line) and achieved dry cell weight concentration (▲)

Productivity reached a value of $3.8 \text{ gDCW l}^{-1} \text{ h}^{-1}$ after 49 h, which was obviously higher than the productivity of the linear feeding profile. This value could be further increased, if a higher

inoculation concentration would be increased, as commonly done at industrial scale. A productivity of $4.6 \text{ gDCW l}^{-1} \text{ h}^{-1}$, could be reached if starting OD would be 20 in contrast to the used 0.2.

As shown in Figure 14, the feed was stopped after 25 h. This is the time point, where maximal cultivation volume was reached. The achieved biomass was different to the predicted, which could be explained by the sample taking. The reduction of the cultivation volume by sample taking made it necessary to recalculate the feeding profile. Lower volume and lower overall biomass decreased substrate inflow. This resulted in a lower specific growth rate at the end of the fermentation. Nevertheless, the overall performance of this cultivation was good, which showed that the fed-batch fermentation was the best choice for achieving high cell density.

3.1.3.5 HCDF on glycerol basis

Preliminary shake flask experiments were performed with medium containing glycerol as sole C-source. It was observed that the strain AH22*ura3* was not able to grow in synthetic media containing glycerol as sole C-source. However, protein determination showed product formation and accumulation. Addition of 0.1 g l^{-1} sucrose to supply additional carbon below Crabtree-concentration resulted in a growth that was only driven by sucrose consumption. In contrast, the addition of 50 g l^{-1} sucrose as present in the DS-medium resulted in a 40% higher optical density after 144 h cultivation, than cultivation without glycerol. Subsequent experiments were carried out with different ethanol concentrations to induce oxidative growth. Also here the achieved optical densities were higher, than without glycerol. Interestingly, complete lack of ethanol did not lead to detectable growth.

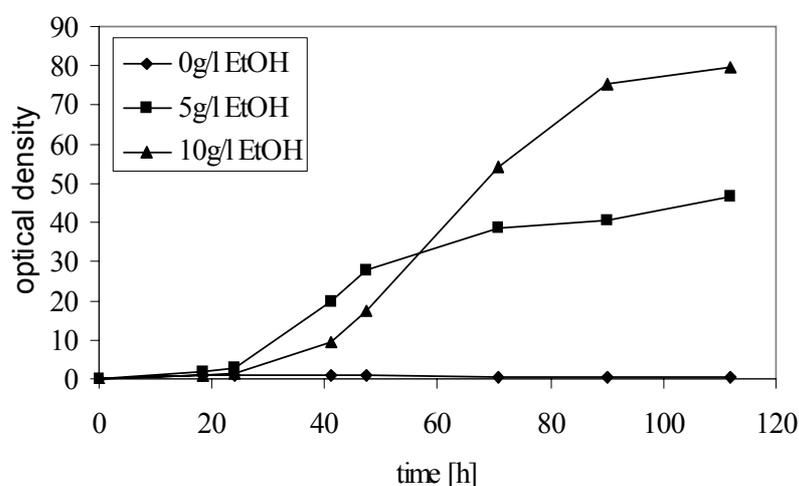


Figure 15: *S. cerevisiae*: Growth on glycerol and ethanol containing media in shake flasks
Cultures grown in shake flasks with DS-medium with glycerol as only carbon source and addition of different concentrations of ethanol

Medium compositions containing different sucrose and glycerol concentrations showed moderate glycerol concentrations of 40 g l^{-1} resulted in higher final optical densities than lower (20 g l^{-1}) or higher concentrations (60 g l^{-1}). Further increase of concentration significantly decreased growth. Complex nitrogen addition (e.g. Na-glutamate or NZ-amines) recovered growth on glycerol and WSP content was increased in comparison to medium supplied with sucrose as sole C-source.

Therefore, fermentation with 40 g l^{-1} glycerol in DS-medium containing 50 g l^{-1} sucrose was performed and led to a biomass of 50 gDCW l^{-1} after 48 h (Figure 16).

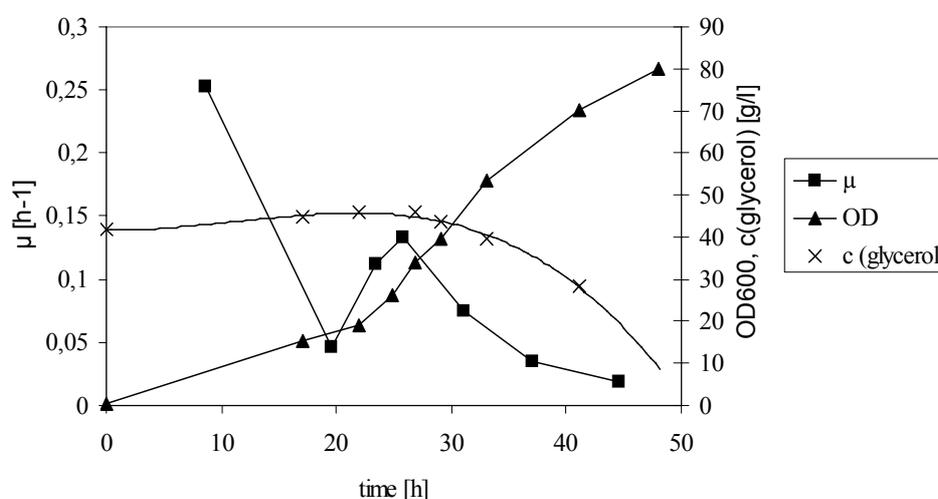


Figure 16: *S. cerevisiae*: Batch fermentation in DS-medium with additional 40 g l^{-1} glycerol. Optical density (▲), specific growth rate (■) and glycerol concentration (x); cultivation was carried out in a 2 l-fermentor.

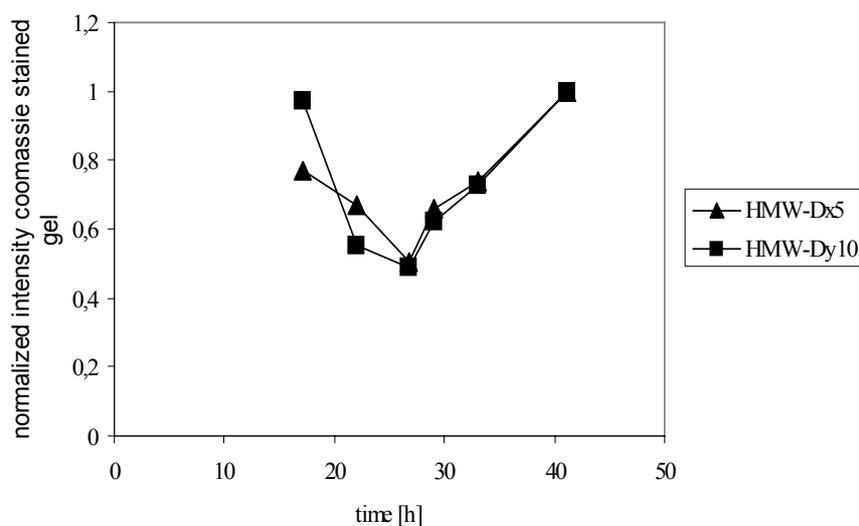


Figure 17: *S. cerevisiae*: HMW-formation in batch-culture in DS-medium with additional 40 g l^{-1} glycerol. HMW-1Dx5 (▲) and HMW-1Dy10 (■) were produced in 2l-batch-cultivation

DCW/OD-ratio changed with glycerol containing medium from 0.4 to 0.62. As shown in Figure 16, after a slightly glycerol production a strong decrease of glycerol concentration occurred, which indicates glycerol consumption. The specific growth rate followed classical progression with diauxic growth behavior. Up to the 20th hour ethanol was produced. Afterwards, ethanol and glycerol were simultaneously consumed.

The evaluation of the formation of HMW-1Dx5 and HMW-1Dy10 showed decreased detectable amounts in the SDS PAGE up to the 30th hour (Figure 17). After that, the increased intracellular concentration of specific proteins, correlated with the decrease of the glycerol concentration in the medium. Therefore, it was assumed that glycerol led to an accumulation of the recombinant proteins.

Cultivation in YEP-G instead of DS-medium resulted in a 7-fold increase of OD, albeit growth was very slow ($\mu < 0.04$). This would also declare the decrease of the long lag phase after the addition of yeast extract.

Because of the good results of applying complex nitrogen sources to glycerol containing cultures a HCDF with two carbon feeds was planned. Sucrose batch fermentation with following sucrose fed-batch fermentation was carried out. A biomass concentration of 130 gDCW l⁻¹ was achieved after 45 h by an H-value of 1.17. A second feed with glycerol (700 g l⁻¹) and Na-glutamate (233 g l⁻¹) was started with a feeding rate of H = 1.10, representing a lowered growth rate. Unfortunately the yeast did not grow on the second substrate (Figure 18). The measured DCW decreased through dilution with second feed medium.

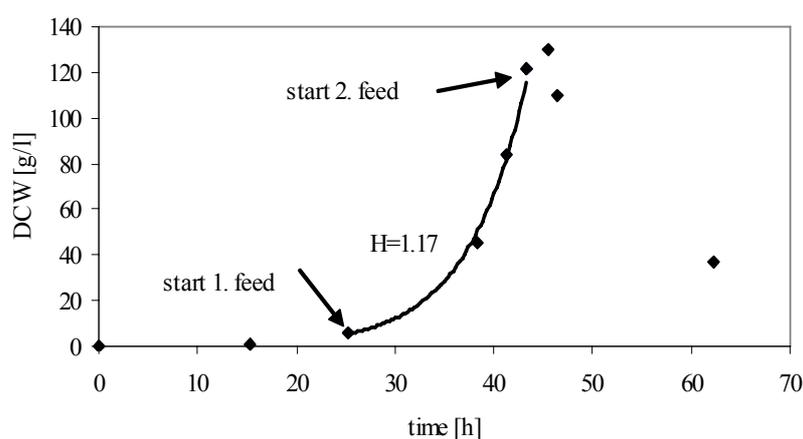


Figure 18: *S. cerevisiae*: Growth (DCW) in HCDF with sucrose (1. feed) and glycerol (2. feed)
Measured DCW (◆) and predicted DCW (—) with H-value of 1.17

To evaluate the growth on glycerol as sole carbon source, batch fermentation with 150 g l⁻¹ glycerol and complex nitrogen sources (10 g l⁻¹ Na-glutamate; 20 g l⁻¹ NZ) was carried out.

Here extreme slow growth was observed. Growth started at low specific growth rate ($\mu=0.05 \text{ h}^{-1}$), but further decreased during the following 100 hours to 0.02 h^{-1} . Because of the slow growth cultivation was terminated after 115 h.

3.2 Optimization of downstream processing

3.2.1 Adaptation of Osborne-fractionation

The determination of the composition of the proteins in wheat is carried out classical with an extraction called Osborne-fractionation. Here the proteins are extracted successively with water, saline solution and 70% ethanol. The residuum can be solved with reducing agents and alcoholic solvents. The proteins of interest are found in the ethanol extract (gliadins) and in the residuum (glutelins). In order to adapt the extraction fast and simply to the recombinant proteins in *S. cerevisiae* and to extract only the alcohol-soluble proteins, a simplification of the classical extraction was performed. Here the proteins were precipitated after the cell disruption with an excess of alcohol and extracted then with an alcohol-water-mixture and reducing agents. Precipitation at the end of the extraction recovered the WSPs from the mixture and offered a further purification step.

Due to the changed host system and different protein contamination, an adaptation of the extraction was necessary. The different storage proteins differentiate, as recognizable from the Osborne-fractionation, in its properties, in particular the alcohol solubility. From that an investigation was necessary for every product. LMW6 and modLMWa3 differs only in a few amino acids, however, equal behavior could be assumed.

Parameters for first precipitation are: kind of alcohol, alcohol-lysate-relationship, alcohol concentration and temperature

Parameters for extraction are: kind of alcohol and reductant, reductant amount, alcohol-pellet-ratio, alcohol concentration and temperature

Parameters for last precipitation are: kind of alcohol, alcohol concentration in the mixture and temperature

2-propanol represents the best extracting agent and is needed in lower amount compared to ethanol (personal message from [E. Hinzmann](#)). Therefore, 2-propanol was used for all following experiments. The precipitation represents an important step in the extraction because the volume to be extracted is reduced strongly. Through simple centrifugation it was not possible to pellet all solid fragments and solved proteins in short time. Because low temperature is beneficial for precipitation, it was set to 4°C. First parameter that was analyzed was the alcohol-lysate-ratio for precipitation before extraction. The concentration of the

precipitation reagent was kept at 100% to reduce dilution. Ratio was varied from 0.25 to 5 for different lysates. Loss of specific protein is presented in Figure 19.

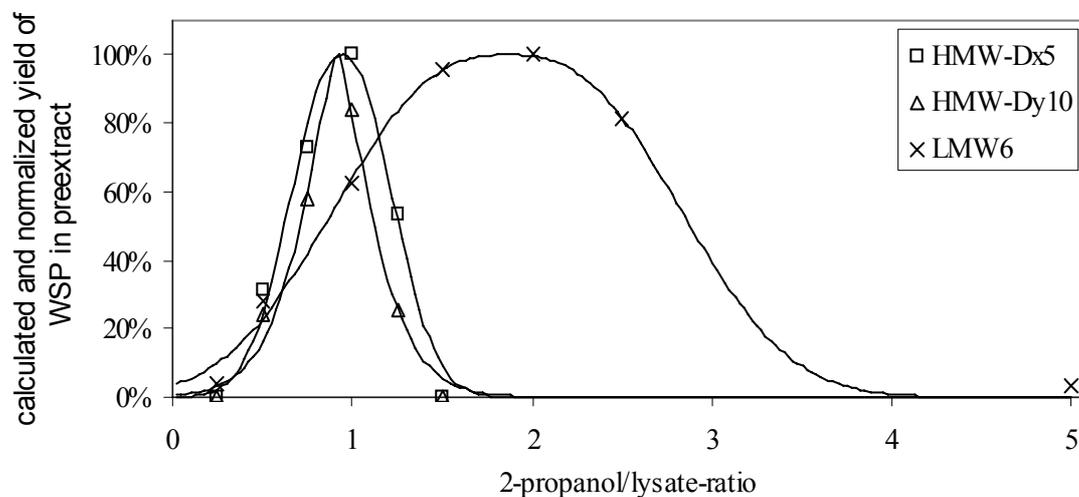


Figure 19: Osborne-fractionation: Influence of 2-propanol/yeast-lysate-ratio on precipitation of WSPs
WSP content in supernatant after precipitation normalized to maximal values

Optimal ratios for the efficient precipitation without loss of recombinant protein were both, very low, below 0.5 and very high, for LMW6 at least 4 and for HMW 1.5.

Low ratios had to be preferred, because of its low 2-propanol-amount. With decreasing alcohol amount the increase in host protein contamination could be observed. A ratio of 0.5 was therefore optimal for precipitation for all WSPs and could be performed for all subunits, even if expressed in one host.

Next parameter, that was investigated, was the percentaged 2-propanol amount in the extraction buffer. To achieve a defined concentration in the mixture, the different lysates were previously precipitated. The normalized yield from the different extraction mixtures are represented in Figure 20. Here it is visible, that there was a strong correlation between HMW-1Dx5 and HMW-1Dy10. LMW6 was extracted in a smaller concentration range and good results could be obtained from 30 to 60% (v/v), but 60% (v/v) was preferred because of the contamination with host proteins at lower amounts. HMW-subunits were extracted at high yields in a broad range (50%). The highest yield could be determined around 40% (v/v) 2-propanol. If extraction buffer was directly given to the lysate, condition changed. Extraction buffer was diluted with lysate and optimal concentration was shifted as represented for LMW6 for 10% (v/v). This extraction method was accompanied by higher 2-propanol requirements and contamination with host cell proteins was increased. Nevertheless, this could be used for the easy detection of WSPs also in ATPS.

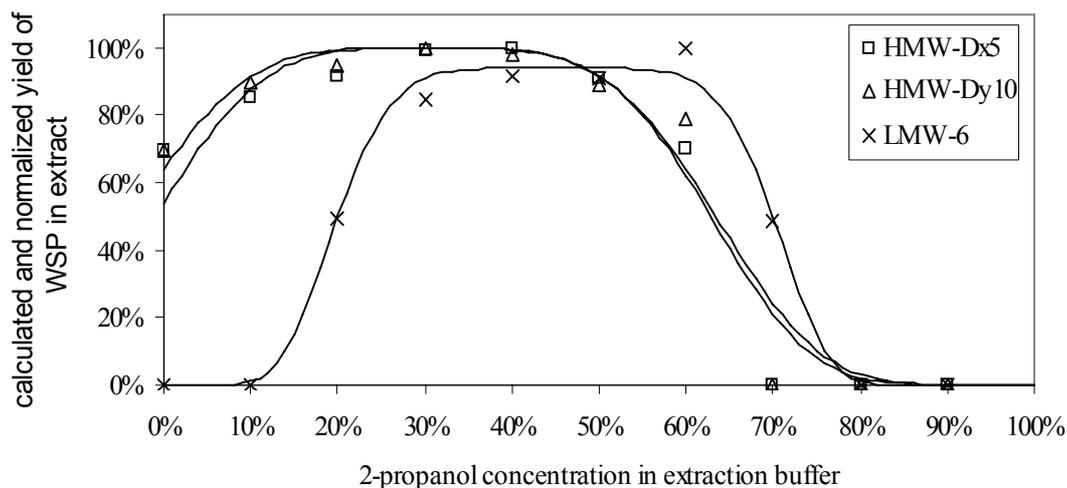


Figure 20: Osborne-fractionation: Influence of 2-propanol concentration on extraction of diff. WSP from yeast

Extraction was performed at different concentrations of 2-propanol in the extraction buffer, quantification was performed by western blot (LMW) and coomassie stained gel (HMW)

The following important parameter was the extraction buffer/precipitate-ratio. Therefore, a defined amount of lysate was precipitated as previous confirmed and extraction buffer volume was varied. Figure 21 gives information about the achieved yields. Here is to be recognized, that, as expected, with increasing volume of extracting agent the extraction yield increased. Due to the logarithmic process the necessary ratio can be restricted. An increase to more than a factor of three did not appear reasonable, since the additional protein yield and the protein concentration were decreased.

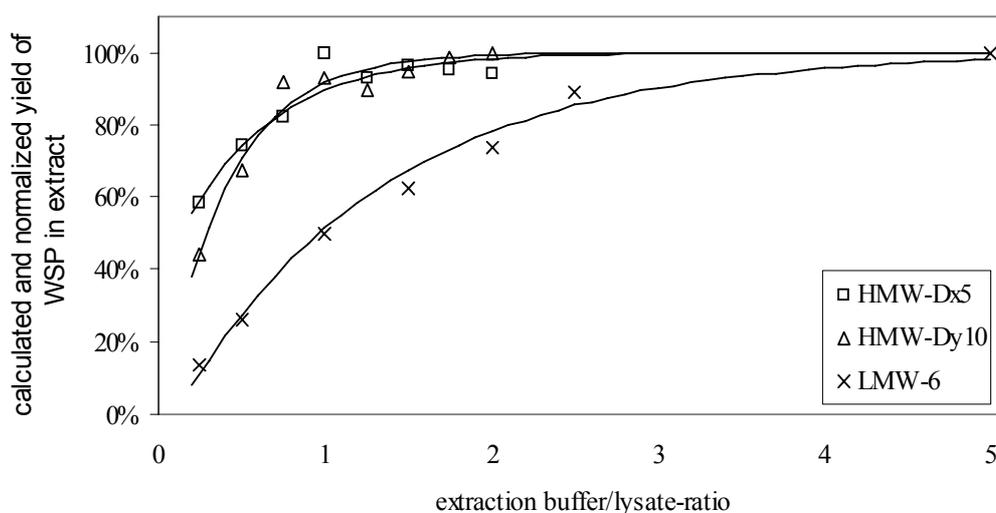


Figure 21: Osborne-fractionation: Influence of buffer/yeast-lysate-ratio on extraction of diff. WSP from yeast

Extraction was performed with different extraction buffer/lysate-ratio; quantification was performed by western blot (LMW) and coomassie stained gel (HMW)

HMW-subunits showed a higher solubility in the extraction buffer. Lower affinity of LMW6 was accompanied by higher necessary volume-ratio. Factor two seemed to be sufficient in all cases. Higher loss of protein was observed for LMW6 than for the HMW-subunits.

Last step in the purification of WSPs was the precipitation to get rid of the alcohol mixture and to further purify WSPs by removing host cell proteins. As in the previous sections 2-propanol was used at different amounts.

Table 6: Osborne-fractionation: Influence of 2-propanol concentration and temp. on precipitation of diff. WSP

Quantification was performed by western blot (LMW) and coomassie staining (HMW), normalized to highest value of precipitation of each WSP

2-prop. concentration in mixture	Normalized yield of WSPs after precipitation			
	HMW-1Dx5 at 4°C	HMW-1Dy10 at 4°C	LMW6 at 25°C	LMW6 at 4°C
50%	11%	12%	2%	100%
60%	20%	26%	22%	92%
70%	92%	97%	34%	99%
80%	100%	100%	46%	88%
90%	95%	93%	100%	68%
95%	44%	33%	97%	60%

Table 6 shows that an increase of 2-propanol-concentration via 100% 2-propanol addition led to higher precipitation rates. HMW-subunit precipitation decreased with exceeding 80%, whereas LMW6 precipitation could be further enhanced at 25°C. Elevation for execution at 4°C could be obtained for lower 2-propanol contents. For effective precipitation all WSPs should be treated with 70% 2-propanol at 4°C.

An interesting observation was made, when HMW-extracts with different content of 2-propanol in extraction buffer were precipitated without further addition of 2-propanol. Figure 22 shows the surprising results.

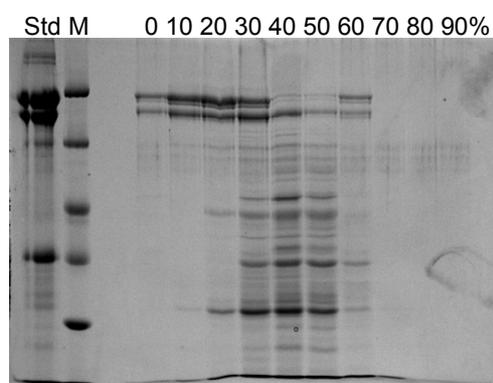


Figure 22: Osborne-fractionation: Influence of 2-propanol concentration precipitation of HMW from yeast

Gel was Coomassie stained and HMW-subunits were extracted with different 2-propanol concentrations and precipitated at 4°C without further addition of 2-propanol

As already shown, the extraction was optimal for HMW-subunits between 20 and 40% (v/v) 2-propanol and 1% DTT. Cooling at 4°C and following centrifugation showed precipitation between extract concentrations of 10 to 30% (v/v). Highest yield with lowest host protein contamination was obtained for 20%. If only HMW has to be separated this procedure would be with lowest 2-propanol usage. And precipitation is initiated only by cooling.

3.2.1.1 Common extraction of HMW- and LMW6-glutenins

The extraction of the different wheat storage proteins significantly differed, but a compromise for extract all WSP in one extraction could be generated without big loss of WSPs. Extractions in which all four different wheat storage proteins are supposed to be simultaneously isolated were represented through following protocol:

Precipitation:

Add to lysate to 25% (v/v) of a 100% 2-propanol, mix

Centrifugation: 10' at 9000rpm at 4°C (lower speed for easy resuspension)

Extraction:

Resuspend pellet with 40-45% (v/v) 2-propanol and 1% (w/v) DTT

Extraction for 1 h at 60°C, when appropriate resuspend again

Centrifugation: 10' at 100% speed at 40°C (to avoid precipitation)

Precipitation:

Shift of concentration of 2-propanol through addition of 100% 2-propanol up to 80 %

Centrifugation: 10' at full speed at 4°C (enhance precipitation)

The buffering to lower pH values was accompanied by a lower host cell protein contamination and higher specific protein amount. New extraction method compared to the classical one exhibit lower loss of specific protein during first precipitation, here the removal of host specific proteins was strongly enhanced. This consequently led to lower host cell protein contamination in extract and in last precipitation. As a result, the requirement for chemical compounds (e.g. 2-propanol) could be significantly reduced by the new extraction method.

3.2.2 Aqueous two-phase system (ATPS)

ATPSs are commonly used to purify proteins from heterogeneous mixtures or lysates. It has a great potential for replacing expensive and time consuming purification steps in industrial productions. ATPSs can combine harvesting, cell disruption and ATPS-purification in one step. Unfortunately, because of its high number of influencing parameters, the specific partition of proteins is not predictable so far. Its application is commonly based on extensive testing of parameters influencing separation properties. Detailed description of ATPS is given in the “Appendix”.

3.2.2.1 Preliminary experiments

To verify whether the application of ATPSs is suitable for the isolation of recombinant WSPs from yeast lysate, LMW6 was chosen for preliminary experiments. It could be detected in broad range and at very low amounts using western blot detection. Independent partition and detection of LMW6 from lysate interaction could be best demonstrated with the use of purified LMW6.

Distribution of purified WSP in ATPS

A solution enriched in LMW6 (50 μ l, 10 mg ml⁻¹ protein containing 25 % of LMW6 protein) was applied to 20% (w/w) PEG 1500/ 7.5% (w/w) K₂HPO₄ pH 9. After two-minute of phase development, samples were taken from the different phases and detected by western-blot (Figure 23).

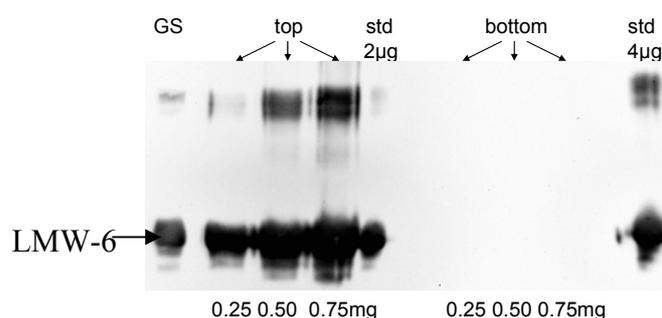


Figure 23: ATPS: Distribution of purified LMW6 in a defined phase system

Western blot detection of LMW in a gel; Lane 1: glutenin standard, lane 2-4: top-phase of three different systems with three different LMW6-protein amounts, lane 5: 2 μ g LMW6-standard, lane 6-8: bottom phase, lane 9: 4 μ g LMW6-standard

Phase separation occurred very quickly and was not accelerated by centrifugation. It was proven, that only the PEG-rich upper phase contained detectable LMW6-protein (Figure 23). This indicated suitability of WSP recovery by selected ATPSs.

Distribution of lysate in ATPS

Before the partition of total protein was investigated, the influence of aqueous two-phase system components on the Bradford-assay was examined. Protein samples with contents of 4, 8 and 20 mg of BSA were analyzed. No significant influencing could be found by PEG 2000, potassium phosphate, Triton X100, PMSF and combinations of it (data not shown).

30% (w/w) lysate was added to systems containing 10% (w/w) PEG 2000 / 10% (w/w) K_2HPO_4/KH_2PO_4 (pH7) and 12% (w/w) PEG 2000 / 12% (w/w) K_2HPO_4/KH_2PO_4 (pH 7). Samples were taken after phase separation and analyzed for overall protein content. Quantification was performed according to Bradford. Despite the different volume ratios and different turbidity of phases, an insignificantly different protein concentration between the phases could be observed. Total protein determination revealed an approximately regular distribution in the tested systems. There were indeed deviations in the concentrations in the different phases. However, the influence of the volume ratio of the phases was considerably higher.

Influence of phase forming components on specific protein detection

At the beginning of the investigation of ATPS the protein samples were taken from the formed phases and directly mixed with sample buffer. After the reduction and denaturation, the samples were put onto the gel. However, salt and PEG were suspected to have big influence on gel run and made it occasionally impossible to detect distinct bands. Therefore, PEG 300 and PEG 1500 with concentrations up to 40% (w/w) were tested for the influence on SDS-PAGE and western blot detection. PEG showed no significant influence on extraction, gel separation and detection. The influence of different salts (K_3PO_4 , K_2HPO_4 and KH_2PO_4) up to 40% (w/w) accompanied by different pH values was investigated. Extreme pH values like 4 and 13 (KH_2PO_4 and K_3PO_4) showed disappearance of bands or led to smeared lanes, respectively.

The big influence of salt and pH on detection of specific proteins led to the introduction of a buffered alcoholic extraction for the quantification of the WSPs in the different phases. Multi-linear regression showed reduced influence of the pH value, but significant influence of the concentration of the salt onto the detectable LMW6 amount. The established extraction was not longer influenced by pH. The new established procedure resulted in lower host cell protein content in the extract and in higher content of specific protein (HMW-1Dx5 and HMW-1Dy10). The quantification could be improved and reproducibility and reliability was increased.

3.2.2.2 Influence of different parameters on ATPS separation

In the following sections the influence of different parameters on specific protein partition is reflected. Following parameters have important influences on phase separation and distribution of biomolecules:

- temperature
- pH-value
- kind of polymer
- kind of salt (charge of salt ions)
- polymer molecular weight and molecular weight distribution
- concentration of the phase forming components (PEG, salt, lysate, additives)
- target molecule properties (e.g. charge, size, solubility and hydrophobicity)

Due to decrease the effort of testing all different parameters, some of them were set to constant values: The temperature for phase formation, which has very great influence, was set to room temperature (25°C) and the choice of system forming components was restricted to PEG/salt-systems. Polymer/polymer-systems were not investigated, because of its low suitability for large scale due to its high price.

Influence of centrifugation on ATPS partition

Viscosity and interfacial tension have great influence on partition speed. Different concentration of lysate in ATPS changes viscosity. Separators or centrifuges are commonly used to enhance the phase development after intensive mixing.

Systems with only small differences in composition were chosen to show importance of centrifugation for complete phase separation. 10% (w/w) PEG 2000 / 10% (w/w) K_2HPO_4/KH_2PO_4 (pH 7) loaded with 20% (w/w) yeast lysate was able to form a two-phase system after 5 min of 1000 x g, whereas 12% (w/w) PEG 2000 and 12% (w/w) K_2HPO_4/KH_2PO_4 (pH 7) was even not completely separated after 120 min. Centrifugation should therefore run at higher speed for sufficient time. In swing out rotors 10 min at 4000 x g showed complete phase separation for all tested systems.

Influence of the positive charged ion of system salt on ATPS partition

Systems containing 30% (w/w) PEG 1000 and 5% (w/w) of ammonium sulfate, sodium sulfate and magnesium sulfate were compared at pH7 due to investigate the influence of the positive charged ion of the system salt on the partition and distribution of lysate. 20% (w/w) lysate containing recombinant LMW6, HMW-1Dx5 and -1Dy10 was used to verify partition in ATPSs. No difference was observed for these conditions. Not only the volume ratio was

similar, but also the specific protein amount in each phase. As expected, the influence of the cation in the salt can be neglected.

Influence of the negative charged ion of the system salt on ATPS partition

Systems containing 30% (w/w) PEG 1000 and 5% (w/w) of ammonium sulfate or potassium phosphate were compared at pH 7 due to investigate the influence of the negative charged ion of the system salt on the partition and distribution of lysate. 20% (w/w) lysate containing recombinant LMW6, HMW-1Dx5 and -1Dy10 was used to verify partition in ATPSs. A higher K-value could be observed for phosphate containing systems. Because of the possibility to control the pH value with combinations of K_3PO_4 , K_2HPO_4 and KH_2PO_4 , phosphate salts show higher suitability for ATPS than others.

Influence of neutral salt and additives on ATPS partition

It was reported that **neutral salt** addition can influence partition in that way, that addition causes elimination of electrostatic effects in favor of hydrophobic effects (Huddleston et al., 1991). Because of the high hydrophobicity of the WSPs, this might improve distribution significantly. Therefore, different amounts of NaCl (1.5; 2.9; 5.8 and 8.8% (w/w)) were added to 10% (w/w) PEG 2000/ 10% (w/w) K_2HPO_4 at pH9 as well as 12% (w/w) PEG 2000/ 12% (w/w) K_2HPO_4 at pH9 and 50% (w/w) lysate, respectively (Figure 24).

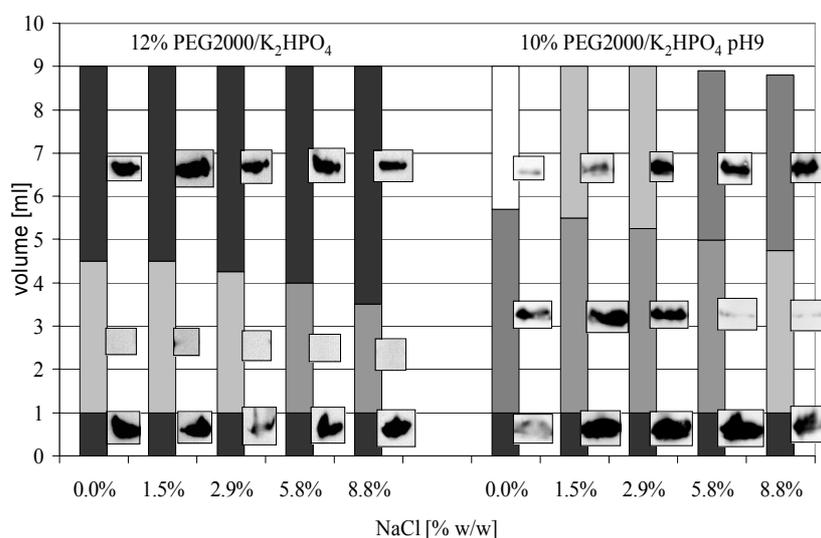


Figure 24: ATPS: Distribution of LMW6 from yeast in two ATPS at different neutral salt addition (NaCl) Bars represent volume ratios of phase systems; intensity of the shadowing shows approx. turbidity; boxes indicate signal of LMW6 in the different phases, determined by western blot detection.

NaCl addition affected the volume ratio of the different ATPS. It rose when NaCl-content was increased. However, partition was influenced differently for different system composition.

10%-system showed different turbidities as well as different LMW6 distribution, whereas 12%-system only showed different volume ratio, but LMW6 was enriched in top phase before NaCl was added. It can be assumed that top phase directed partition is increased at higher NaCl concentrations.

Potassium thiocyanate, described as an neutral salt additive, which increases pH difference between the two phases and thus increases electrostatic driving force (Xu et al., 2001), were tested as a second neutral salt. 5% (w/w) KSCN were applied to 20% (w/w) PEG 1500 / 7% (w/w) K_2HPO_4 pH9 and 50% (w/w) lysate. Unfortunately, it influenced the top phase directed partition negatively, which was consistent with the results from NaCl addition.

Also the addition of **urea** is known to enhance solubility and partition at certain concentration (Ramsch et al., 1999; Ramsch et al., 2000). In the used system the addition of 5% (w/w) urea had an opposite effect in comparison to KSCN and lead to a slight increase in partition coefficient, as formerly described.

Despite the positive influence of neutral salts, addition led only to a changed distribution between top and bottom phase, but pellet associated LMW6 was not affected.

Influence of lysate amount on ATPS partition

Due to the fact that lysate itself shows phase forming properties and amount of lysate influences viscosity, different lysate amounts were added to 12% (w/w) PEG 2000 / 12% (w/w) K_2HPO_4 pH9 .

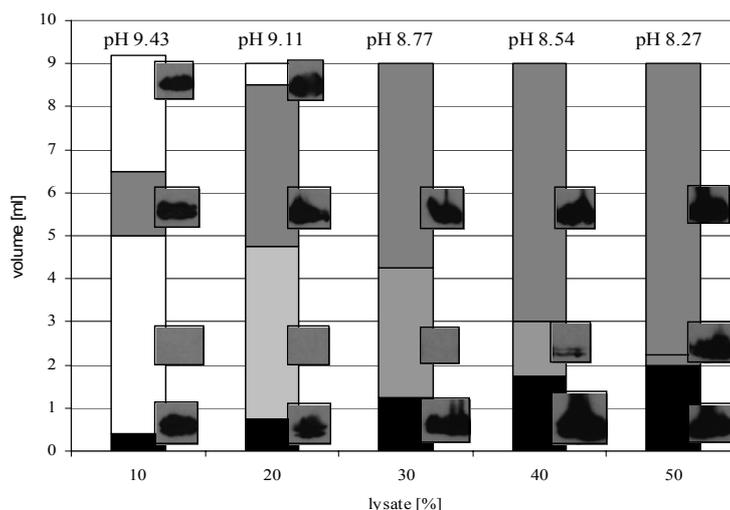


Figure 25: ATPS: Distribution of LMW6 from yeast in ATPS loaded with different lysate amounts (%)
Bars represent volume ratios of phase systems; intensity of the shadowing shows approx. turbidity; boxes indicate signal of LMW6 in the different phases, determined by western blot detection.

Increasing lysate amount resulted in different effects (Figure 25). Phase volume ratio increased with increasing lysate amount. Turbidity of the phase indicated by grey scale obviously increased, too. For low lysate amounts, LMW6 was detected mainly in the top phase and in the pellet of the bottom phase. At low lysate amounts no LMW6 could be detected in the bottom phase. Higher biomass addition seemed to increase LMW6 concentration in the bottom phase, but this was probably detected, because sample taking was hindered at higher viscosity.

Influence of pH value on ATPS partition (71, 98, 97-99, buffered 107,108,109,123)

The pH value represents one of the decisive parameters for the distribution in ATPSs. It decides for the location of the binodal in the phase diagram and restricts this way the working area (Figure 26). Increasing pH values resulted in a binodal shift directed to the origin. Furthermore, the system pH influences the net charge of the proteins, which further influences its phase partition. As shown in Figure 26, different systems (●) were chosen for the investigation of the influence of pH and location in the phase diagram on partition.

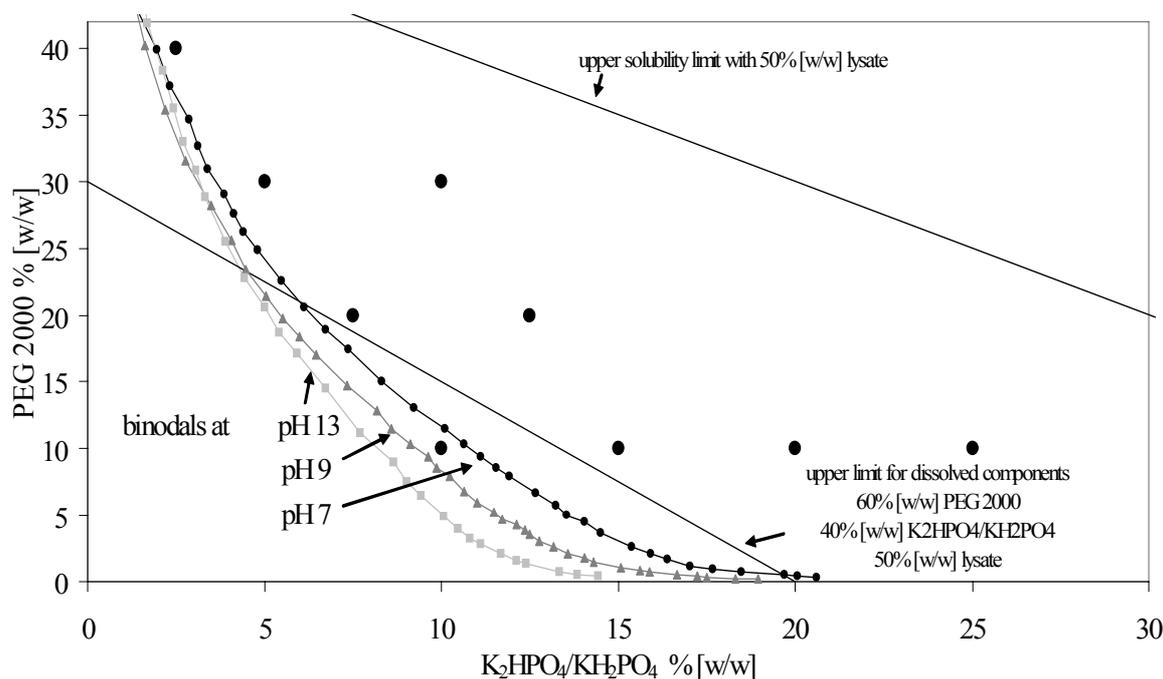


Figure 26: ATPS: Influence of the pH value on phase development
Position of systems chosen for investigation of pH-influence (●); binodals at different pH values were shown at different grey grade

Binodals were determined through cloud point method (see section “Material and methods”). With the binodal also operating point moved by changing the pH. Systems marked with (●) in Figure 26 were shown after complete phase separation in Figure 27.

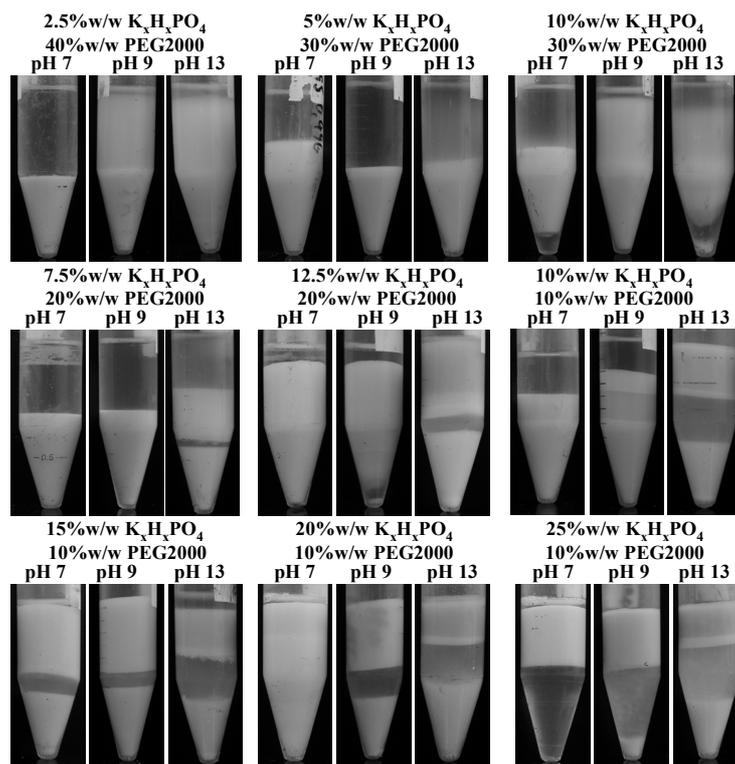


Figure 27: ATPS: Phase development of systems with different composition and pH values
Composition and pH values are given on top of the images

Although system composition remained unaltered at different pH for the selected systems, the distribution of visible lysate particles (cell debris, intracellular compounds) and volume ratios were changed. This could be explained by dependence of tie-line length on pH value. Increased pH and salt content led to top phase directed partition. Volume ratio changed drastically if cell debris was shifted to the top phase.

To examine the distribution of LMW6 at different pH values, LMW6 containing lysate was applied to a PEG2000/potassium phosphate system at pH values of 4, 7, 9 and 13. The yield of LMW6 extractable in top phase and the partition coefficient were calculated and are shown in Figure 28. It is obvious that increasing pH value improves the yield and the partition coefficient (K). The end could be drawn, that high pH values would be recommended for the purification. Unfortunately, the host cell protein concentration and the amount of cell components were also strongly increased. At pH 13 it could be microscopically shown that only cell wall components remain in the saline bottom phase (Figure 34). With that the purification would be inadequate, because of intracellular vesicles found in top phase. An interesting observation was made for WSP extraction. Unbuffered extraction at low pH or without addition of salt showed very low amounts of contaminants in the SDS-PAGE. This could be explained by different solubility of different components at low pH values.

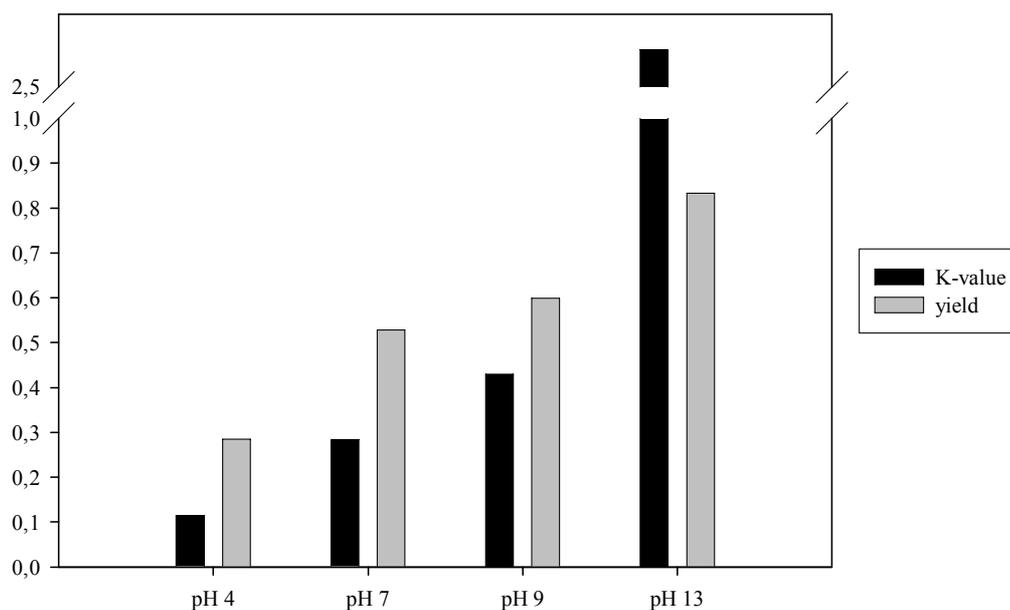


Figure 28: ATPS: Partition (K-value) and yield (see page 39) of LMW6 from yeast in a PEG2000-system at different pH values

Influence of polymer molecular weight on ATPS partition

Molecular weight of PEG (PEGs MW) is another main decisive parameter in ATPSs. The higher the PEGs WM, the lower the distance to the origin (Figure). That means that the working area grows with increasing PEGs MW and thus decreasing concentrations of phase forming substances are necessary. A disadvantage of high molecular weight is the increasing viscosity and longer mixing and separation time. Investigation of PEGs MW influence was performed with molecular weight ranging from 1000 to 6000 Da (Figure 29).

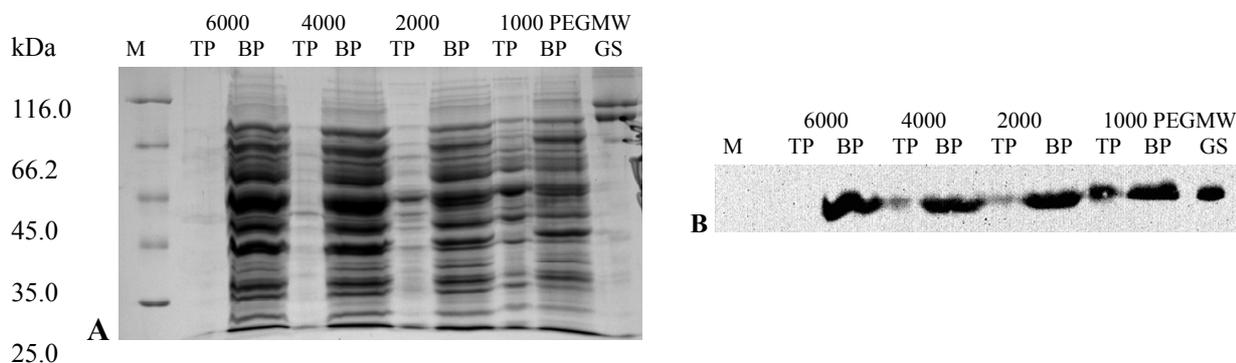


Figure 29: ATPS: Influence of PEG with different molecular weights on distribution of LMW6 from yeast Coomassie stained gel (A) and western blot detection (B) of top (TP) and bottom (BP) phases of ATPS; M: marker; GS: glutenin standard

Because of the different position of the binodal, a graphical estimation for obtaining equal binodal distance was performed. 50% (w/w) lysate and 30% (w/w) PEG 1000, 2000, 4000 and 6000 was used, whereas 7.2, 6.1, 5.2 and 4.8% (w/w) K_2HPO_4 pH9 was used, respectively.

The lower the PEGs molecular weight was the higher was the protein content in the top phase. This is not only referred to the specific protein (LMW6), but also to the host cell protein. The contamination was increased in parallel.

Influence of phase components concentration on ATPS partition

Three systems were chosen for determination of the influence of distance from binodal and of phase forming capacity of lysate. To all systems, below (8% (w/w) PEG 2000/ 8% (w/w) K_2HPO_4/KH_2PO_4 pH7) , close to (10% (w/w) PEG 2000/ 10% (w/w) K_2HPO_4/KH_2PO_4 pH7) and above (12% (w/w) PEG 2000/ 12% (w/w) K_2HPO_4/KH_2PO_4 pH7) the binodal 20% (w/w) (1g), 40% (w/w) (2g) und 60% (w/w) (3g) lysate were added. In Figure 30 the volume distribution and the western blot detection of the LMW6 is presented. The system below the binodal showed, as expected, no phase development and LMW6 was found in the supernatant as well as in the pellet. The dark labeled pellet increased obviously with the lysate amount. The composition of the system close to the binodal resulted in a phase separation, where the top phase was clear and contained nearly equal amounts of LMW6 as bottom phase and pellet. Higher biomass load shifted the system border and the pellet volume up. The system above the binodal developed an intermediate phase that appeared as precipitation in the top-phase. Here LMW6 was enriched in the top-phase, whereas the salt rich bottom phase was found to be LMW6-free. This observation strengthens the applicability for using ATPSs. The pellet-associated LMW6 concentration was equal for all systems and indicated non-soluble or cell debris associated LMW6. The system above the binodal seemed suitable to enrich LMW6 in the top phase and separate product from cell debris, with moderate loss of LMW6 that was present in the pellet.

The experiment was repeated with different lysate resulting from disruptions lacking or containing Triton X100 for enhanced membrane solubility and PMSF for reduced protein degradation. No difference was observed, even if lysate stock was frozen or fresh. Different detergents (Triton X100, Triton X114, Tween 20 and Tween 80) and 2-propanol were added after cell disruption to improve partition of pellet-associated LMW6-subunit. Unfortunately the addition led either to additional phases or to different volume ratios, but not to an improved protein partition. When detergents were added during cell disruption additional phase development in ATPSs disappeared, but partition was again not improved.

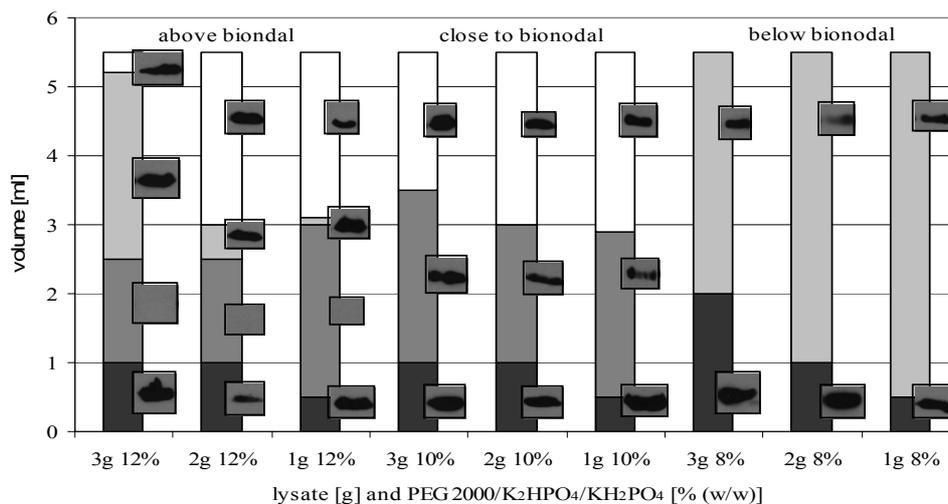


Figure 30: ATPS: Distribution of LMW6 from yeast in three different systems
Bars represent volume ratios of phase systems; intensity of the shadowing shows approx. turbidity; boxes indicate signal of LMW6 in the different phases, determined by western blot detection.

PEG as only phase forming substance

Several experiments led to the assumption that a phase separation would be possible by directly mixing lysate with PEG. The advantage would be the use of only one system component and thus lower costs for chemicals. Preparation of such systems and analysis revealed, however, a bottom phase directed partition, which was not preferred.

3.2.2.3 Increase of WSP solubility

Because the low effectivity of the selected ATPSs was caused by the loss of specific proteins in the bottom located pellet, efforts were done to improve the release of WSPs from the pellet or increase its solubility.

Second extraction

Commonly, extraction is limited by the volume of the extraction solution and the equilibrium concentration. Repeated extraction should lead to further top phase partitioned LMW6, if such a limitation has occurred. Therefore, a 10 g system containing 11.1% (w/w) PEG6000 and 8.5% (w/w) K₂HPO₄/KH₂PO₄ (pH 7) was prepared and mixed with 20% (w/w) lysate. After phase separation samples were taken from each phase and pellet was again mixed with phase forming components as in the first system.

In the first extraction LMW6 was distributed equally between the different phases. The second extraction did not lead to a distribution of the pellet associated LMW6 into PEG-rich phases. It could not be detected in the top phase. The assumption that LMW6 is found in

pellet due to incomplete extraction can be excluded. Despite this observation, volume ratio of developed phases was similar between the systems.

The extraction was repeated with different systems in 50 g scale for HMW as well as LMW subunits. Top phase was finally loaded with a low amount of host cell protein, but no specific protein could be detected. These observations showed the complete distribution of product into the phase of higher affinity.

Application of detergents

Detergent based extractions are effective primary steps for isolation of amphiphilic biomolecules. The application could lead to a release of so far inaccessible WSPs. Therefore, 2-4% (w/w) SDS (anionic detergent) was mixed with the lysate before applied to the system 20% (w/w) PEG1500 / 5% (w/w) K_2HPO_4 pH9. The results, however, showed that as higher the SDS content in the mixture, as lower the LMW6 amount detectable in the top phase. Even the use of 5% (w/w) Tween 20 (non-ionic), hexadexyltrimethylammoniumbromide (cationic) and Zwittergent 3-10 (amphotheric) did not improve solubility of LMW6 or phase separation. The complete substitution of the phase forming polymer PEG by Tween 20 led to a detergent-based (non-ionic) ATPS. 20/8; 15/9; 10/10; 5/11; 20/9; 15/10; 10/11; 5/12; 20/10; 15/11; 10/12 and 5% (w/w) Tween 20 / 13% (w/w) K_2HPO_4 pH9 were produced, respectively. 20% (w/w) lysate was added and after phase separation phases were analyzed for LMW6 content by western blot detection. Phase separation occurs, but unfortunately no release or solubilization of LMW6 was shown. The major amount of LMW6 was still found in the cell debris pellet.

Temperature induced detergent based phase separation

Hydrophobic proteins can be extracted by the use of detergents, which is commonly done for membrane proteins. Therefore detergent based ATPS could be used to recover hydrophobic WSPs from yeast lysate. Triton X114 represents a thermoseparating polymer (for further information see "Appendix"), which possesses a cloud point temperature around 22°C. 2-8% (w/w) Triton X114 was applied to form a temperature induced two-phase system with yeast lysate contents up to 92% (w/w). Components were vigorously mixed and heated up to 30°C. Phase separation occurred and phases were separated by decanting. Phases were analyzed for HMW- and LMW-subunits by coomassie staining and western blot detection, respectively. Neither HMW- nor LMW-subunits could be detected in the top phases. WSPs were only found in the bottom phases. Because host cell proteins were found in the top phase, this

method would be suitable to lower the contamination of host cell proteins. The purification from cell debris was not possible.

Application of reducing agents and alcohols

S. cerevisiae is able to form disulfide bridges between proteins. If the WSPs form intracellular covalent links, reduction could lead to the release from cell debris pellet. Therefore, lysates were preliminary treated with 1% (w/w) DTT or β -mercaptoethanol before added to 20% (w/w) PEG 1500/ 7% (w/w) K_2HO_4 pH9 up to 50% (w/w). The necessary temperature for effective reduction was determined at 60°C. The procedure showed no progress to previous experiments. In contrast, high temperature treatment led to formation of precipitates that were favored bottom phase partitioned.

The addition of alcohols (methanol, ethanol, 2-propanol and acetone), as described as additives (Johansson and Kopperschlager, 1987), led to similar effects as reductive agents. Alcoholic precipitations led to increased bottom phase partition.

Influence of cell disruption

Although the disruption of cells was always microscopically controlled, a prolonged cell disruption may improve the release of specific proteins from intracellular vesicles. A sequence of different disruption times was performed (0, 5, 15 and 30min). Afterwards the produced lysates were applied to an ATPS (20% (w/w) PEG1500/ 8% (w/w) K_2HPO_4 pH9) and phases were analyzed for WSP content (Figure 31).

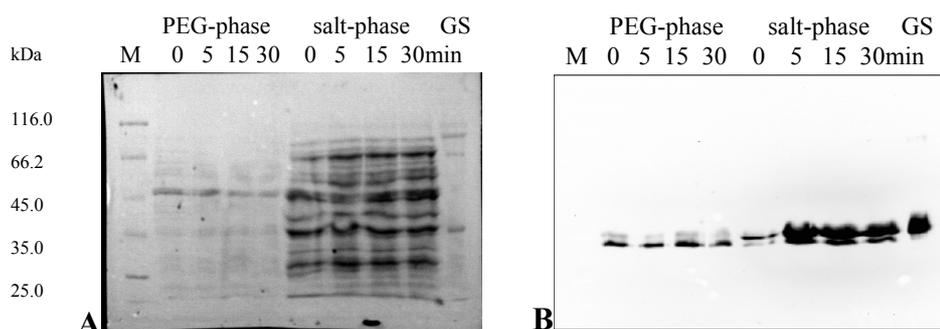


Figure 31: ATPS: Influence of cell disruption time on distribution of LMW6 from yeast Ponceau S staining (A) and western blot detection (B) in PEG-phase (top phase) and salt-phase (bottom phase); M: marker; GS: glutenin standard

It is striking that the specific protein amount in the PEG-rich top phase was not affected by the cell disruption. Even if no cell disruption was performed, the detected LMW6 concentration in top phase was comparable to that from disruptions. That led to the assumption that top phase associated protein was extracellularly located before cell disruption was actually performed. Detected protein amount in the salt phase was released and detected

after disruption. No increase of LMW6 amount after extending disruption time longer than 5 minutes could be observed. This showed sufficient cell disruption after 5 minutes of treatment.

Immunofluorescence for determination of intracellular localization of WSPs

Immunofluorescence was performed for the determination of intracellular product localization. AH22ura3 Δ pmt1 YpADH LMW6 was cultivated in DS-medium containing shake flasks. Samples were taken after 17, 45 and 65 h. The fluorescence of LMW6 was microscopically determined.

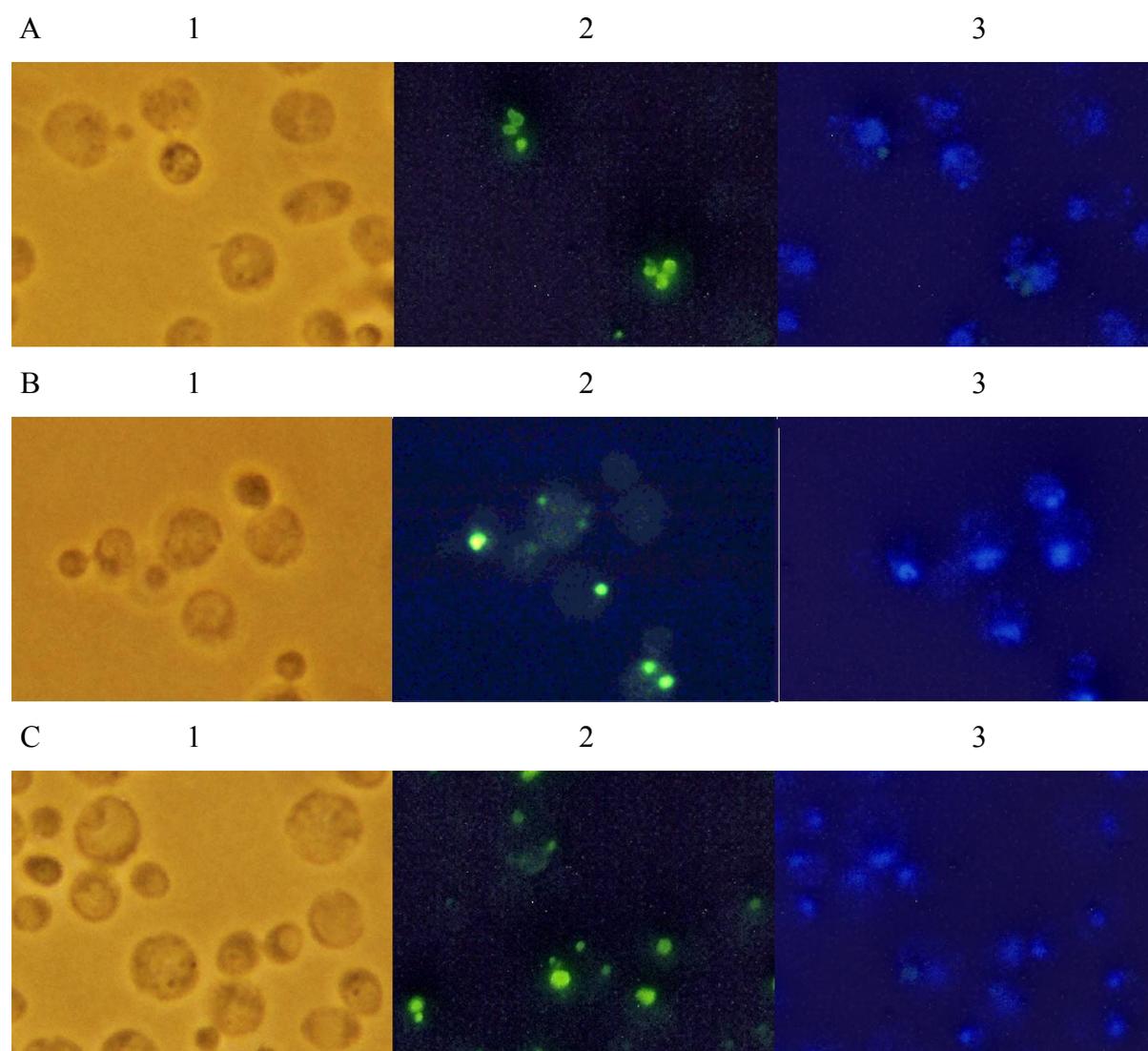


Figure 32: *S. cerevisiae*: Cellular localization of LMW6 by immunofluorescence microscopy
Phase contrast (1), FITC labeled (1) and DAPI labeled (1) cells of AH22ura3 Δ pmt1 YpADH LMW-6 after 17h (A), 41h (B) and 65h (C)

Microscopically pictures showed cells that exhibits strong compartmented FITC-fluorescence. As shown in Figure 32, fluorescence was not nucleus associated and not spread in the

cytoplasm. The structures imply endoplasmatic reticulum associated protein localization. Also the cytoplasmic aggregation, as found for inclusion bodies would be possible.

3.2.2.4 Selection of suitable ATPS

Two different ATPSs were found to be suitable for further analysis. The first system (18% (w/w) PEG 2000 / 7% (w/w) K_2HPO_4 pH9, 50% lysate) was chosen, because of the high concentration of detected LMW6 and the complete lack of solid particles in the top phase. Unfortunately this system was accompanied by a moderate loss of specific WSP in the bottom phase. As shown in Figure 33 the PEG 2000/ K_2HPO_4 and the PEG 1500/ K_2HPO_4 systems are suitable for LMW6 extraction from yeast lysate.

In order to show suitability of the chosen systems for HMW partition, HMW containing lysates were applied to the ATPSs. Unfortunately, it turned out to be not successfully for HMW extraction. Detectable amounts were only found in the bottom phase of both tested systems, as shown in Figure 33C.

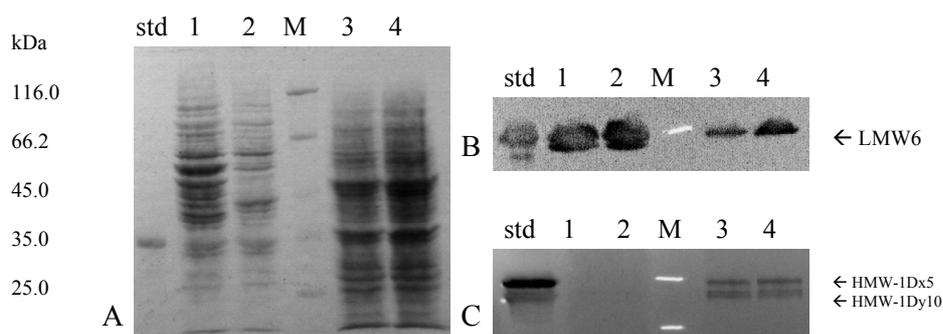


Figure 33: ATPS: Distribution of total protein and diff. WSP from yeast in two selected* phase systems (A) total proteins were stained with ponceau S; (B) western blot detection of LMW6; (C) coomassie staining of HMW-1Dx5 and -1Dy10; std: glutenin standard, 1: top phase (TP) PEG 1500; 2: TP PEG 2000; M: protein molecular weight marker (configured); 3: bottom phase (BP) PEG 1500 4: BP PEG 2000; * selected systems are PEG 1500/ K_2HPO_4 and PEG 2000/ K_2HPO_4 (pH 9)

A similar behavior was presented for the use of PEG with lower molecular weight. LMW6 protein was found in detectable amounts in the top phase, whereas HMW protein could mainly be detected in the bottom phase. When salt concentration was increased to a point where cell debris was distributed to the top phase HMW protein could be detected in top phase.

As a second system 15% (w/w) PEG 300 / 15% (w/w) K_3PO_4 pH13, 50% lysate was chosen, because of nearly full partition of WSPs into the top phase. The resulting system showed a yellow top phase with medium density and a white bottom phase with similar density, but lower viscosity. Microscopic pictures revealed a top phase containing intracellular vesicles,

but the more interesting observation was made in the bottom phase. This was completely loaded with broken cell walls and no other particles could be found, as shown in Figure 34.

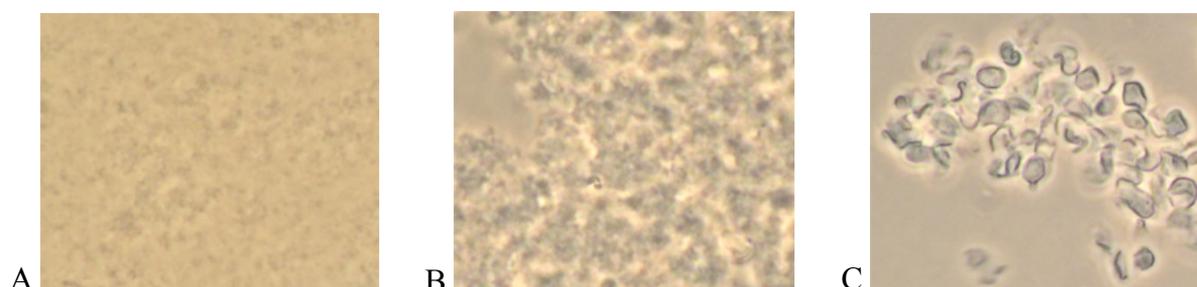


Figure 34: ATPS: Microscopic images for localization of cell particles and cell walls in different phases (A) undiluted top, (B) undiluted intermediate and (C) 1:10 diluted bottom phase

If the selected system was allowed to separate on work bench for 30 min or was centrifuged for 2 min at 1000 x g, phase separation was almost completed and top phase appeared homogeneous and was not splitted in two phases. This could relieve the recovery of the top phase for further process steps.

The top phase was free of big cell wall components, but contained cell debris particles. Nucleic acid amount in the different phases was first analyzed through ethidium bromide staining. Here PEG led to fluorescence and hindered the quantification. This could be used for the quantification of PEG in systems free from nucleic acids. Therefore, nucleic acids were extracted and separated by agarose gel electrophoresis (Figure 35).

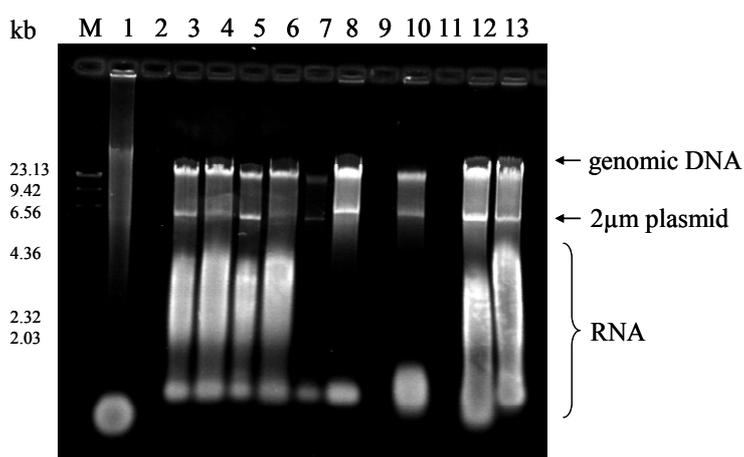


Figure 35: ATPS: Nucleic acid partition in systems of different composition ethidium bromide stained nucleic acids separated in agarose gel; M: lambda DNA/HindIII marker, 1: top and 2: bottom phase of PEG300/K₃PO₄ pH13; 3: top and 4: bottom phase of PEG2000/K₂HPO₄ pH9; 5: top and 6: bottom phase of PEG2000/K₂HPO₄/KH₂PO₄ pH7; 7: top and 8: bottom phase of PEG1500/K₂HPO₄ pH9; 9: top and 10: bottom phase of PEG1000/MgSO₄ pH7; 11: top and 12: bottom phase of PEG1500/KH₂PO₄ pH4; 13: lysate

Unfortunately, the ethidium bromide staining showed that only the top phase contained nucleic acids. Comparison to other systems revealed that it is indeed possible to reduce

nucleic acids in the top phase through bottom phase directed partition, but WSP partition was changed similarly.

Recovery of product (second system)

Generally, ATPS extraction takes place in two steps. After the separation occurrence of the first system, the specific protein enriched phased is removed and further purified. The first system should remove cell debris, a majority of host cell proteins, lipids and nucleic acids. A second system is employed to transfer the enriched product from PEG-rich top phase to the salt rich bottom phase. Here further purification and removal of lipids, nucleic acids and colored pigments takes place. The product-free polymer phase can thus be recycled to reduce process costs. If the second system is performed with an appropriate salt, a precipitation through concentration shift could be employed to concentrate the product and remove salt phase or suitable chromatography can be coupled directly.

Second system development was forced by the addition of salt solution consisting of 40% (w/w) K_2HPO_4/KH_2PO_4 (pH 7). The pH shift should direct PEG phase associated proteins to the bottom phase. Stepwise addition to the top phase of the first system created several systems, which fulfilled the requirements. However, no specific protein could be detected in the bottom phase of the developing systems.

In order to investigate a salt that would be applicable for hydrophobic interaction chromatography (HIC), 40% (w/w) NH_4SO_4 solution was stepwise added to the PEG2000 phase. Volume ratio decreased by successive addition, but no WSPs were detected in the bottom phase.

Due to further increase the stringency of bottom phase partition, PEG of higher molecular weight was and lower pH were employed. Intensive testing revealed the system PEG6000 K_2HPO_4 . Here WSPs were completely bottom phase partitioned. Solid cell particles and host cell proteins were also found in the bottom phase.

In order to verify the purification from lipids, extraction from the different phases was performed. Direct determination of the weight of the extracted lipids was not possible, because PEG again disturbed the measurement. Because of that, thin layer chromatography (TLC) was employed to visualize the different lipid contents. 20 and 10 μ l extract were alternately put onto the TLC-plate (Figure 36). The plate was illuminated by UV-light and image was made without the use of filters. Similar to the partition of nucleic acids also the partition of lipids is mainly directed like that of the specific WSP. Major amount, as shown in Figure 36, were found in the top phase of the first system. Some fractions were also found at the cell wall-rich bottom phase. In the second system partition behavior is more pronounced.

Here all lipids were found in the bottom phase, which means contamination of the bottom partitioned WSPs.

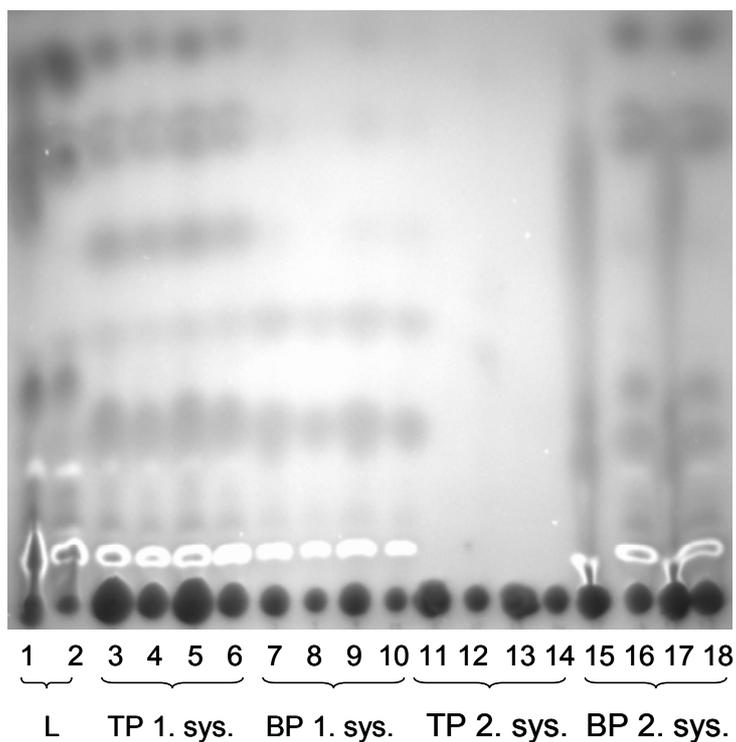


Figure 36: ATPS: Lipid partition in two subsequent selected systems

Thin layer chromatography of lipid extracts from different ATPS phases; extracts from 1&2: lysate, 3, 4, 5 & 6: top phase and 7, 8, 9 & 10: bottom phase of the first system (PEG 300/K₃PO₄ pH13); 11, 12, 13 & 14: top phase and 15, 16, 17 & 18: bottom phase of the second system (PEG6000/KH₂PO₄ pH4); even numbers: 10µl, odd numbers: 20µl of extracts

Negative partition of were shown in both nucleic acid and lipid analyses. Purification from contaminating lipids and nucleic acid was not possible by the chosen systems.

Summary

The influence of media composition on growth and recombinant protein production was investigated. It was found that only a small number of carbon sources especially sucrose were suitable in minimal medium. Although glycerol was functional in complex medium and accumulation of recombinant proteins was observed, minimal medium at selective conditions did not lead to sufficient growth. DS-medium was limited in nitrogen for complete carbon utilization, which could be overcome by supplementation of glutamate or NZ-amines. Further medium components (mineral material, vitamins) were varied in concentration and approximately demand was determined. Process parameters (temperature, pH, pH-regulation, maximal feeding rate) were determined for subsequent HCDF. These were carried out at different modes (linear feeding, predetermined exponential feeding) and different carbon sources (sucrose, glycerol). Highest biomass formation was gained for linear feeding with sucrose (ca. 200 gDCW l⁻¹), whereas highest biomass productivity was gained for exponential feeding with sucrose (3.8 gDCW l⁻¹ h⁻¹ after 49 h). Growth on glycerol was found to be too slow for the use in HCDF.

The adaptation of the classical Osborne-fractionation for the extraction of recombinant WSPs from yeast led to a number of parameters influencing the purity and the yield of proteins. Especially the 2-propanol/yeast-lysate-ratio for previous precipitation, the 2-propanol-concentration in the extraction buffer and the 2-propanol content for final precipitation were found to be important. Values were determined to increase protein yield by parallel decrease of necessary 2-propanol amount. Although values were different for LMW and HMW-subunits, a method was found to extract both WSPs without big loss at high purity. The pH value represents another important factor that decides for solubility and purity. Here further investigations are necessary.

The parallel establishing of an ATPS for food suitable extraction of WSPs revealed a variety of parameters that influences partition of recombinant proteins in ATPS. Kind and amount of polymer and salt, molecular weight of the polymer, pH value, temperature, biomass load, influence of neutral salt and temperature induces phase formation were investigated. Partition of recombinant WSPs in yeast lysate either contributed to high loss of recombinant WSPs or was accompanied by high contamination of host cell proteins. Reasons for that could be found in hydrophobicity, intracellular aggregation or formation of insoluble particles that favored particle partition in contrast to protein partition. Nevertheless, ATPS represents an alternative extraction method with future opportunities.

4 Discussion

The main aim of this work was the increase of recombinant WSP output from the yeast *S. cerevisiae*. The practical work focused on the two parts: upstream and downstream processing. In the part “Upstream processing” it could be demonstrated that medium conditions and cultivation parameters positively influence recombinant protein production and biomass formation of the yeast *S. cerevisiae*. A very high cell density could be achieved in which WSP output was increased several-fold.

In the part “Downstream processing” the classical Osborne fractionation could be successfully adapted to the recombinant WSP recovery from yeast. A simple alcoholic extraction could be established that were able to extract LMW as well as HMW-subunits at high yields with lowest usage of extraction solvent. Furthermore, an aqueous-two phase system should be established to find an alternative extraction method that contributes to food-suitable production. A variety of ATPSs were screened and selected for the separation of WSPs from yeast lysate in one step.

4.1 Upstream processing

4.1.1 Optimization of media composition

The media composition not only influences growth rate and biomass yield, but also the production of recombinant proteins. The determination of preferred medium components is indispensable to successfully perform HCDF. Preliminary investigation revealed conditions at which HCDF and high specific recombinant protein formation could be obtained. Because growth and recombinant product formation seemed to be correlated for most of the used medium compositions, high biomass formation was accompanied by high total recombinant protein production. If shake flasks were used for preliminary investigations, high concentrations of fermentable carbon sources were found to induce inhibition of growth by **ethanol accumulation**. This observation was also described earlier (Brom, 1986). Therefore, carbon source contents equivalent to 50 g l⁻¹ sucrose were found to be the highest no inhibition inducing concentration. Ethanol tolerance is strongly dependent on the strain, the intracellular lipids (You et al., 2003) and influenced by the calcium and magnesium concentration in the medium (Ciesarova et al., 1996).

S. cerevisiae is commonly able to grow on a variety of carbon sources. The influence of acetate, ethanol, fructose, galactose, glucose, glycerol, maltose, sorbitol and sucrose on growth and WSP production were evaluated in shake flasks. Despite all of the tested carbon

sources runs into glycolysis, only fructose, glucose and sucrose showed sufficient growth rate and recombinant protein production. Although equivalent carbon concentrations were supplied, **sucrose** showed the highest specific growth rate, maximal obtained biomass and highest total recombinant protein production. These results were not expected, because glucose is commonly used as preferred carbon source. Also the extracellular cleavage of sucrose would imply an additional bottleneck for the uptake. But because *S. cerevisiae* possesses a sucrose uptake system (Stambuk et al., 2000) and sucrose can be intracellularly metabolized, which was shown by hexose transport null mutants (Batista et al., 2004), it is not surprising that higher μ_{\max} could be obtained for sucrose than for glucose and fructose in equimolar concentrations (Orlowski and Barford, 1991). Interesting in this context is the published paper from Verstrepen et al. (2004). They reported that glucose and sucrose are “hazardous fast-food” for industrial yeast, because of their negative effects (e.g. incomplete fermentation, poor maintenance of vitality).

All other tested **carbon sources** showed slow or even arrested growth, especially if inorganic nitrogen was present in the medium. Some C-sources (e.g. maltose) are taken up by active transport, which corresponds to higher energy requirements and results in ca. 25% lower biomass yield compared to glucose (Weusthuis et al., 1993).

Ethanol, glycerol and acetate represent **non-fermentable** metabolic byproducts of the yeast *S. cerevisiae*, which are only consumed as long as no fermentable C-sources are available. Induction of oxidative enzymes and cellular remodeling (e.g. changes in peroxysomes and mitochondria) becomes necessary to assimilate them through gluconeogenesis (McCammion, 1996). This might be a further reason why non-fermentable C-sources show slow growth, especially after transition from fermentable C-sources.

Despite slow growth on glycerol as sole C-source, high recombinant protein production could be observed in several experiments. This could be a reason for the reported low internal amino acid pool at growth on glycerol as sole C-source (Martinez-Force and Benitez, 1995). Why glycerol does not permit growth as sole C-source in synthetic medium cannot be declared. Growth on complex medium as YEP-G could be achieved. This let assume an absence of a unknown growth factor (Kim et al., 2000) only essential under non-fermentable conditions and maybe synthesized after long cultivation. Although AH22 was not able to sufficiently grow on glycerol as sole carbon source in minimal medium, glycerol represents a carbon source that shows no catabolite repression, can be used for HCDF at high biomass productivity up to $5.5 \text{ gDCW l}^{-1} \text{ h}^{-1}$ (Eugene Raj et al., 2002) and recombinant protein accumulation (Wong et al., 2002). It is cheap, can be supplied at high concentrations without

ethanol accumulation, Y_{XS} is higher than for other tested C-sources and simple feeding strategies are applicable as shown for *Yarrowia lipolytica* (Kim et al., 2000). If growth inhibition of glycerol could be overcome, cheap raw-glycerol, a by-product of bio-diesel production, could be used (Papanikolaou et al., 2002).

The growth defects, which could be observed for the used yeast strain on several carbon sources (e.g. glycerol and acetate), could be explained by the “construction” of the strains. Laboratory strains were commonly produced by undirected mutagenesis and selection for special properties, e.g. auxotrophy (Hashimoto et al., 2005). Other mutations might also have been occurred but remained undetected. Therefore, mutations hidden in permissive conditions could be a reason for the observed growth defects. Industrial production is commonly performed in complex media. Auxotrophic marker present in the used strains prohibits the use of complex media. The usage of wildtype strains and marker that can not be complemented by medium components would increase growth, recombinant protein production and process opportunities. Another “growth defect” was shown by the linear growth at the “exponential growth phase” in shake flask cultivations and the much lower specific growth rate compared to cultivations in the fermentor. Reasons for that could be found in the strongly limited oxygen entry in shake flasks, which is even more effective for non-fermentable C-sources. Oxygen limitation superimposes the effects of medium composition and could result in wrong interpretations. It could be partially compensated by using baffled shake flasks on circular shaker. Nevertheless, oxygen limitation is always present at moderate cell densities in shake flasks, as shown for *E. coli* (Losen et al., 2004). A further improvement would be the application of 48-well microtiter plates. High oxygen transfer rate of $0.28 \text{ mol l}^{-1} \text{ h}^{-1}$ could be achieved (Kensy et al., 2005). It combines the advantages of high-throughput 96-well microtiter plates and Erlenmeyer flasks.

Fermentable C-sources were found to decrease the **cell size** of *S. cerevisiae*, which makes them highly suitable for HCDF. Small cell size results in improved mass transfer and substrate supply, which will also lead to higher biomass formation. The reduction of cell size was found to be regulated by SFP1 and resulted not only from lower growth rate, but also from the oxidative metabolism (Cipollina et al., 2005).

For ethanol and glycerol containing cultures the addition of **complex organic nitrogen** (glutamate and NZ-amines) significantly improved the growth rate and specific recombinant protein production. The lowered observed degradation of recombinant proteins after transition to stationary phase by addition of complex nitrogen led to the assumption of insufficient nitrogen content in DS-medium. Improved initial growth rate and shortened lag phase could

be explained by lower metabolic burden for amino acid synthesis. The increased biomass and recombinant protein expression by provision of preferred amino acids was also described by [Gorgens et al. \(2005\)](#). In this connection it is interestingly to note that in marker experiments it was shown, that almost no CO₂ was produced by the assimilation of glutamate, because almost all glutamate was directed into protein biosynthesis ([Albers et al., 1998](#)).

Also single amino acid addition (e.g. arginine and asparagine) led to higher recombinant protein content after entering stationary phase. A similar effect was reported by [Gaczynska et al. \(2003\)](#), who supposed that an addition of arginine- or proline-rich peptides has a protease inhibitory effect. This is further consistent with the reported inhibitory effects of amino acids and peptides on biomass autolysis ([Nekliudov et al., 1994](#)). The addition of PMSF as protease inhibitor showed analogous results, which strengthen the lower proteolytic degradation theory. But also the addition of other nitrogen sources (ammonium, glutamate, protein hydrolyzates) showed similar effect, which also could represent a nitrogen limitation complementation.

NZ-amines led to improved growth and product formation compared to CA. Despite the fact that CA contains higher percentaged amino acids amount and a higher amino nitrogen/total nitrogen-ratio, NZ contains higher amounts of rare amino acids (e.g. tryptophan, methionine) and leucine (complementing leucine auxotrophy).

At different media composition (e.g. C-sources, vitamins) different expression levels and different progressions could be observed for the detected WSPs. The observed differences in could be an effect of the different genetic constructs. HMW-subunits are synthesized by integrated constructs, whereas LMW-subunits are synthesized by integrated and plasmid related constructs. Although basic construction of expression cassettes is equal, gene dosage affected by copy number can cause severe differences. Also the selection pressure for plasmid maintenance could have been varied during cultivations, at which integrated constructs were not being affected.

Carrying out several experiments revealed that the determination of DCW was presenting a difficulty. A simple method to determine DCW represents the measurement of the optical density. Although it is fast, easy performed and commonly applied in microbiological laboratories, results have to be verified. Because indirect scatter light measurement depends on medium (refractive index), cells (size, shape and surface structure) and spectrometer device, OD/DCW-ratios is variable and it is not surprising, that the measurements differed up to 30%. The performed shake flask cultivations were helpful for investigations at low biomass level, but for high cell density, such measurements are not reliable, since respiro-

fermentative-ratio, growth rates, substrate biomass yield (Y_{XS}), oxygen demand and intake of substrate may completely change. However, shake flask based measurements can give valuable hints for substrate preferences, but only trends for actual substrate demand.

4.1.2 Process parameter

The **pH value** of the cultivation media and its progression were strongly dependent on medium composition in the performed experiments. For instance ammonium as sole nitrogen source caused rigorous decrease of medium pH, whereas glutamate uptake was accompanied by a pH increase. Therefore, parallel provision of ammonium and glutamate made pH-regulation unnecessary, because of its pH balancing character. The uptake of bases or release of acids will actively decrease medium pH and vice versa. The consumption of one NH_4 (e.g. ammonium sulfate) is accompanied by the release of one H^+ into the medium. Therefore, ammonium hydroxide (25%-ammoniac) could be successfully used for pH compensation at large scale cultivation. Nitrogen limitation and high fermentor volume changes could be excluded, because of its strong basic character. Furthermore, increasing osmolarity of the medium, which will take place by addition of salts (e.g. KOH, sodium or potassium phosphate), could be circumvented.

Optimal medium pH for growth and product formation was found at pH 4.5. Because preculture medium pH was 5, reduced adaptation requirement could be a reason for the observation. Nevertheless, higher observed oxygen demand, which was found for pH 3 and 6.5, could not be explained by reduced adaptation requirement. Reasons for it could be found in increased metabolic activity for proton-pumping (Onyeaka et al., 2003), drifting of respiro-fermentative-ratio or different nitrogen assimilation at different pH values (Vicente et al., 1998). Reduced biomass yield could be explained by the loss of carbon for energy production (proton-pumping).

S. cerevisiae is commonly able to grow in a very large pH-range. This extends for most of the strains from pH 2 to pH 8, whereas optimal range extends from pH 3 to pH 6.5 (Crans et al., 2004). The dependence of pH value on growth can be explained by different effects: different uptake rate of media components due to the different dissociation state (especially iron and copper) (Serrano et al., 2004), low hydrogen ion concentration causes lack symptoms and high concentrations seems to be inhibitory or even toxic. The cell-external pH value can be varied over a wide range without strongly affecting the cell-internal pH value, because *S. cerevisiae* possesses of proton-pumps, which actively control proton concentration.

Also the ethanol tolerance was mainly affected by medium pH. Low medium pH, developed during ethanol formation phase, caused two effects: inhibitory ethanol concentration was

produced and ethanol tolerance was decreased by lowered medium pH. Buffer capacity (150 mM potassium-phosphate-buffer) and high initial pH value (pH 7) were not sufficient to avoid growth inhibition. Only higher buffer capacities or other nitrogen compositions of the cultivation medium decreased drastic conditions. The phenomenon of increasing minimal persisted pH value dependent on ethanol concentration (< 4 % pH 2.15; at 8.5% pH 3.5) was also described by Bronn (1986).

Beside the factor pH-value, also the feeding rate Highest **H-value** (hourly increase factor) of 1.17 h^{-1} could be obtained for the growth in DS-medium. Higher values resulted in lower biomass formation and thus lower volumetric productivity. Above the determined value, sucrose accumulation above Crabtree-concentration led to ethanol formation and thus to a reduced biomass substrate yield coefficient (Y_{XS}).

The determination of the optimal H-value is severely dependent on the strain and the media composition. Here also the temperature is decisive for a maximum product formation. It was set for the experiments at 29°C . For complex media (e.g. molasses medium) higher H-value of 1.25 h^{-1} could be obtained for *S. cerevisiae* (Bronn, 1986).

4.1.2.1 HCDF

HCDF was performed by initial batch fermentation followed by a highly concentrated feed of carbon and nitrogen at different feeding modes: linear (3.1.3.3) and exponential feeding profiles (3.1.3.4).

A Linear feeding profile, which is accompanied by permanently decreasing specific growth rate, was chosen to reduce oxygen demand at high cell density, because provision of oxygen enriched air should be avoided for cost reasons. 40 g l^{-1} glycerol was added to the batch medium to prevent strong carbon limitation before start of feed.

Batch fermentation showed initially increasing glycerol concentration. This indicated osmotic stress situation that led to the biosynthesis of glycerol (Siderius et al., 2000). After sucrose depletion both glycerol and ethanol were concomitantly consumed during batch fermentation. Respecting the enormous solubility of sucrose (approx. 1.5 g in 1 ml H_2O) and glutamate (740 g l^{-1}), 800 g l^{-1} sucrose and 100 g l^{-1} Na-glutamate monohydrate were successfully fed to the fermentor. Despite highest aeration and stirring rate, oxygen was limited after 48 h. Furthermore, almost all detectable substrates were limited at this time point. Only sulfate and ammonium were sufficiently present, because of addition for pH maintenance. Although mentioned limitations occurred, growth could still be observable for further 26 h.

Oxygen limitation was not sufficient to completely trigger alcoholic fermentation. Substrate limitation or occurrence of oscillation led to measured accumulation of substrates, which

were, however, not inhibitory. Also ethanol was produced, but only to a relatively small extent (15 g l^{-1}) compared to high carbon source intake (800 g l^{-1} sucrose). This could indicate insufficient mixing (high local sucrose concentration) or oscillation that also commonly leads to oscillating ethanol concentrations (Xu and Tsurugi, 2006). Because ammonia was present in the medium at sufficient concentrations, nitrogen limitation could be excluded. Complete carbon limitation in contrast to nitrogen limitation has to be avoided, to maintain fermentative capacity and a productive yeast population (Thomsson et al., 2005).

A very high biomass formation was achieved ($OD \approx \text{ca. } 500 \equiv 208 \text{ gDCW l}^{-1}$). Unfortunately redundant measurement of DCW and optical density was only performed up to 120 g l^{-1} . Here a DCW/OD-ratio of 0.41 (0.99 Pearson's correlation coefficient) was determined. At higher cell density only optical density was measured, knowing of the increasing dilution error.

The cultivation was terminated after 75 h, because continued cultivation would have decreased biomass productivity below the highest value of $2.8 \text{ gDCW l}^{-1} \text{ h}^{-1}$. On the other side inoculation at higher cell densities would have increased productivity to $3.3 \text{ gDCW l}^{-1} \text{ h}^{-1}$ by further reducing cultivation time.

The specific heterologous protein formation at HCDF was comparable to low scale fermentation. Therefore, total protein formation was mainly affected by biomass formation. Interestingly the observed specific HMW-subunit formation increased during batch cultivation and decreased during feeding up to 55th hour. After that time point, when oxygen limitation occurred, an increased specific HMW-formation was observed again. Nevertheless, these changes of specific protein formation are of lower significance than total WSP formation by increased cell density, because of the growth coupled product formation.

Despite the linear feeding, **exponential feeding** profile was applied to reach higher productivity, by higher feeding rate. 700 g l^{-1} sucrose instead of 800 g l^{-1} was fed to the fermentor to decrease the high viscosity, observed at the cultivation with linear feeding profile. Because of the high oxygen demand detected at fermentations with linear feeding profile, increased oxygen supply became necessary. Oxygen saturation was kept above 75% by manually changing volumetric inflow of pure oxygen. DCW of 185 g l^{-1} was obtained with biomass productivity of $3.8 \text{ gDCW l}^{-1} \text{ h}^{-1}$ after 49 h. Inoculation of higher cell densities would have increased productivity to $4.6 \text{ gDCW l}^{-1} \text{ h}^{-1}$ by reducing cultivation time. Further increase of biomass output might be obtained, if the maximal working volume of the vessel could be increased, or culture broth could be continuously harvested. Nevertheless, cell density and volumetric productivity were extraordinary high. Such high cell density could only be achieved at 190 gDCW l^{-1} with *E. coli* (Nakano et al., 1997), at 233 g l^{-1} with

Methylobacterium extorquens (Suzuki et al., 1986) at 184 gDCW l⁻¹ with *Bacillus subtilis* (Yong Soo Park, 1992), at 268 gDCW l⁻¹ with *Candida brassicae* (Yano et al., 1985) and at 235 gDCW l⁻¹ with *S. cerevisiae* (Suzuki et al., 1997). Often very high cell densities were only achieved by cell retention systems, which significantly lower productivity.

Recombinant protein production was not investigated for the exponential feeding profile, but in the light of growth coupled product formation, very high specific WSP formation can be assumed.

It was described, that glycerol was successfully used for high biomass production (Eugene Raj et al., 2002). Despite *S. cerevisiae* is able to metabolize glycerol as sole C-source, synthetic DS-medium did not support this behaviour. Therefore, another feeding strategy was chosen, where initial sucrose feeding was followed by **glycerol feeding**. Because both feeds are metabolized by oxidation, long adaptation to new carbon source was not expected. Unfortunately the second feed was too high to measure extending growth on glycerol. However, measured DCW decreased, because of dilution effects. A significant shift of OD/DCW-ratio from 0.41 to 0.6 could be observed. This could be produced through different morphologies of the yeast, through an actual increase of the dry biomass in glycerol containing medium or through smaller cell size.

At biomass concentrations above 100 gDCW l⁻¹ **oscillation** in oxygen saturation and specific growth rate have been observed (Figure 37).

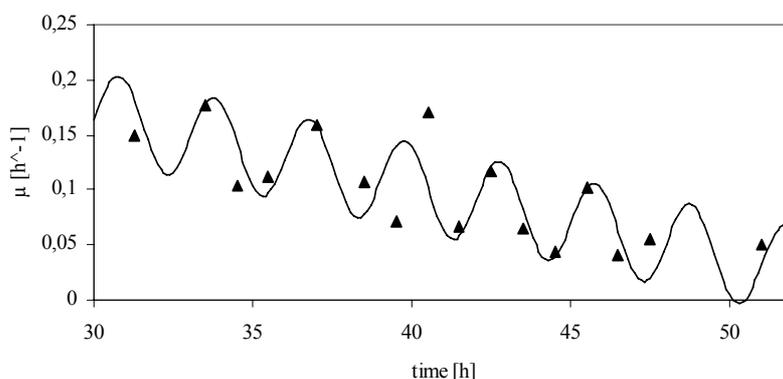


Figure 37: *S. cerevisiae*: Oscillations of specific growth rate (μ) in HCDF with linear feeding profile
At higher cell densities of fed-batch fermentation with applied linear feeding profile growth rate oscillation occurred (3.1.3.3)

Such oscillation have been described in the literature and are presumed to result from several parameters, e.g. substrate addition oscillations (Johnston and Cooney, 2003). Substrate oscillation can lead to a better production and product quality (Bylund et al., 2000). Three different oscillation types are known (i) glycolytic oscillations, (ii) autonomous short-period

oscillations, and (iii) autonomous cell-cycle synchronized oscillations (Hans et al., 2003). Synchronization of cell cycle described by Schlegel (1992) can be caused by nutrition limitation, as happened at HCDF. The determined wavelength of the observed oscillation was in the magnitude of hours, which would prefer cell-cycle dependent of growth related oscillation. Determined equation for describing oscillation represents dependence of specific growth rate (μ) from time (t):

$$\mu = 0,04\sin(2,1t + 6) - 0,0065t + 0,3637$$

4.2 Downstream processing

4.2.1 Adaptation Osborne fractionation

Classical Osborne-fractionation was commonly performed to identify and quantify all proteins of the wheat endosperm. Because only two fractions from the yeast lysate are important (recombinant gliadins and glutenins), the fractionation could be simplified to an alcoholic extraction. The detailed investigation of this step revealed parameters, which had to be considered in order to reduce the loss of specific proteins, increase product purity and also reduce the amount of extraction chemicals. The extraction process was divided in three parts:

- Precipitation of lysate; to reduce extractable volume and the amount of chemicals
- Extraction of precipitate; to recover WSPs from yeast lysate
- Precipitation of extracts; to recover proteins from extraction solution and reduce the amount of contaminating host cell proteins

A low ratio of 0.5 of 2-propanol/yeast lysate resulted in complete precipitation of WSPs by forming a turbid supernatant, which indicates remaining host cell proteins. This represented best ratio, because low loss of recombinant protein was observed and reduction of host cell proteins could be achieved. Higher ratios resulted in loss of recombinant proteins, because of its increasing solubility in the alcoholic solutions. Further increase of ratios above 1.5 for HMW and 3 for LMW-subunits led to clear supernatants and complete precipitation, which corresponds to a complete alcoholic precipitation. This was not preferred, because host cell protein contamination and amount of necessary 2-propanol would increase.

For the extraction of WSP from the precipitate two parameters were important: the concentration of 2-propanol in the extraction buffer and the amount of extraction buffer given to the precipitate. The optimal concentration of 2-propanol in the extraction buffer was different for HMW- and LMW-subunits. HMW-subunits could be sufficiently extracted with 2-propanol concentrations from 0 - 60%, whereas LMW was extracted from 30 - 70%. This could be explained by the higher ratio of hydrophobic amino acids in LMW-subunits (ca.

30%) than in the HMW-subunits (ca. 15%). Fortunately, there was an overlapping range where all tested WSP could be extracted in one step (Figure 20). This extraction behavior was expected, since it was known that WSPs were extractable in alcoholic solutions. Low alcohol concentrations would result in low solubility of hydrophobic proteins, whereas high concentrations would lead to complete precipitation. The influence of the second parameter was also predictable: As higher the extraction-buffer amount as higher the WSP yield. Also here different behavior between HMW- and LMW-subunits could be observed. HMW proteins were extracted with yields of 90% at a ratio of 1 (extraction-buffer/yeast-lysate), whereas for LMW a ratio of 2 was necessary to gain a yield above 80% and 3 for 90%. Because high ratios were uneconomic and would hinder subsequent precipitation, a ratio of 2 was found to be sufficient. That LMW subunits required higher amounts of extraction buffer was not expected, but it could be explained by the higher isoelectric point of LMW-subunits (calculated around pH 9) than of HMW-subunits (calculated around pH 7). Extraction was performed at pH 7, which resulted in a lower net charge for HMW- than for LMW-subunits. Because hydrophobic interactions are increased if charges are reduced, this might be beneficial for HMW extractions.

Final 2-propanol concentration was decisive for the last precipitation step. For the extraction of all tested WSP a final 2-propanol concentration of 70% was useful. But also here HMW and LMW behaved differently. HMW subunits required at least 70% 2-propanol in the final mixture, whereas LMW-subunit could be precipitated at 50% 2-propanol without increase to higher values. Also this could be explained by the higher hydrophobicity of LMW. Lowered temperature, decreased solubility and hydrophobic interactions between different LMW-molecules led to faster precipitation than for HMW-subunits.

An interesting observation was made for the extraction of HMW-subunits. They were successfully extracted and precipitated at 2-propanol concentrations of 10 – 30% in extraction buffer with subsequent cooling from 60°C to 4°C without addition of further 2-propanol. This procedure was not applicable for LMW-subunits, but reduced the extraction effort for HMW-subunits significantly.

Further observations revealed the pH value as an important factor to enhance solubility and reduce host cell protein contamination. Acidic conditions could decrease contaminants solubility, but increase the solubility of WSP. The enhanced solubility of WSPs in weak acids was already described earlier (Truust and Johansson, 1996). Other chemicals that could be used for the extraction of WSP were the alcohols: methanol, ethanol, isopropyl alcohol, n-propanol, the bases: ammonium hydroxide, potassium hydroxide and sodium hydroxide,

and the acids: adipic, ascorbic, aspartic, citric, malic, formic, fumaric, phosphoric, succinic, tartaric, hydrochloric and sulfuric acids and mixtures thereof (Lawhon, 1987; Bassi et al., 1995; Chaundy et al., 1998; Yajima and Katahira, 1998). Despite these agents also the addition of polysaccharides (e.g. pectin) can increase solubility of WSPs (Chaundy et al., 1998). Alternative reductive agents for the extraction of WSPs are sodium hypophosphite (Bauer, 2006), sodium sulfite, sodium bisulfite, sodium metabisulfite or ascorbic acid, that acts as both an acidic and a reducing agent (Bassi et al., 1995). Not only the chosen chemicals are decisive for successful extraction, but also the procedure, which can also be combined, as shown recently (Bauer, 2006). The approach used the combination of 2-propanol extraction and salting out. Precipitation of the WSPs was achieved in the alcoholic top phase by addition of ammonium sulfate.

In the light of the high number of extraction chemicals and reductive agents, extraction of WSPs from yeast lysate can obviously be further improved, but even the optimization of 2-propanol extraction significantly increased the yield of recombinant protein.

4.2.2 Aqueous two-phase systems

ATPSs are commonly performed for the extraction of recombinant proteins (Kepka et al., 2003; Yanagida et al., 2004; Xu et al., 2005) in **two operations**. A first system is used to get the product into a PEG-rich top phase to separate cell debris and the majority of host cell proteins. A second system is applied to recover the product from the PEG-phase and to further purify it from unwanted impurities. High salt content of the last system favors subsequent purification steps like hydrophobic interaction chromatography or precipitation via simple salting out.

A PEG-potassium phosphate system was chosen for phase partitioning during this work, because of its high biocompatibility (Albertsson, 1961). Furthermore, potassium phosphate could be used to set the pH value and keep it constant by its high buffer capacity. The contribution of the anion to phase partitioning was found to be more important than the cation of the phase forming components. Phosphate was found to improve protein partition compared to other anions. This effect was also reported by Huddleston et al. (1991). Anions of higher basicity show higher applicability for ATPS.

After achieving a reliable quantification of WSPs in ATPS has been achieved, the first step for establishing an ATPS was to find parameters that made it possible to separate the WSPs into the PEG-rich top phase, while cell debris remain in the salt-rich bottom phase. Preliminary experiments with **purified LMW6 subunit** and **yeast lysate** confirmed the

expected behavior and showed only top phase directed partition of the WSP and an even distribution of total protein between the phases.

The determination of the binodal was practically performed by the “cloud point method”. But it should be noted that the position of the binodal as well as the complete phase diagram (PD) was dependent on system composition. A displacement of the binodal to the origin of the PD was observed, if the lysate amount was increased, also reported by [Rito-Palomares and Cueto \(2000\)](#), if the system pH was raised and if the molecular weight of the polymer was increased, also described by [Huddleston et al. \(1991\)](#). The influence of lysate on PD could be explained by its phase forming property, which was mainly caused by cellular DNA ([Köhler, 1989](#)). The displacement of the binodal PD to the origin enlarged the working area above the binodal and decreased thereby the required amount of phase forming components. Displacement with growing distance from the origin would decrease the working area and would shift the working points to higher viscosities, which negatively influence phase formation. The working area is not only restricted by the binodal, but also by the tie-line length (TLL). Increasing the distance of a selected system from binodal will lengthens TLL, which is accompanied by an increasing viscosity of the system. High viscosity limited the application of ATPS, because phase separation time is strongly increasing with viscosity.

Several parameters were investigated that further influenced the partition of cell particles and proteins between the phases: The **lysate amount** was varied to find effective concentration for successful partitioning. 50% (w/w) lysate was determined as upper limit and should not be exceeded to limit the influence on viscosity and partition. To produce solved phases and prevent precipitations or occurrence of interface, the protein concentration should be kept low, as previously reported ([Andrews and Asenjo, 1996](#)). Increasing **system pH** led to top phase distribution of LMW6 subunit. This could be explained by the isoelectric point (IP) of these proteins. pH values above the IP led to negatively net charge and resulted in top phase partition ([Johansson et al., 1998](#)). PEG behaves as if it were positively charged ([Huddleston et al., 1991](#)). Unfortunately top phase partition was also found for other contaminating host cell proteins as well as insoluble cell particles. Similar observations were made for decreasing **PEG molecular weights**. Also here a complete top phase partition of WSPs could only be obtained, if parts of cell debris were also found in the top phase (3.2.2.2.7). This effect has been attributed to the rising representation of hydrophilic groups on shorter PEG polymer chains, which reduced overall hydrophobicity ([Hatti-Kaul, 2001](#)). But a more likely explanation is that the excluded volume effects of PEG were increased with the molecular weight. It was reported, that the partition coefficient becomes insensitive to molecular weight

changes above a certain value of molecular weight of PEG (10 kDa in PEG/dextran and 3.5 kDa in PEG/salt-systems) (Huddleston and Lyddiatt, 1990). The positive effect of **neutral salt** (e.g. NaCl) that was reported for the partition of recombinant protein in ATPS (Huddleston and Lyddiatt, 1990; Huddleston et al., 1991; Cunha et al., 2000) could also be confirmed for the selected systems, however, a majority of LMW6 subunit remained in the pellet of the bottom phase.

Several systems have been identified where the WSPs were distributed to a clear PEG-rich top phase without solid cell particles, but high protein loss (>40%) were observed, since a major part of the detected WSPs remained in the cell particle fraction. Increasing top phase directed partition of WSPs by successive raising pH, decreasing molecular weight of PEG or an increase of salt concentration in the bottom phase led always to **bottom phase partitioned WSPs** until cell debris were also distributed to the top phase.

That the observed behavior of LMW6 subunit was not caused by a saturation of the top phase was excluded by a second ATPS, where the LMW6 subunit containing pellet of the lower phase was again mixed with new phase forming components. No further LMW6 subunit could be detected in the top phase (3.2.2.3.1). The high hydrophobicity of WSPs, which results from the high content of hydrophobic amino acids (Truust and Johansson, 1996), and ability to form a network via disulfide-bridges, strengthened the hypothesis of the formation of insoluble agglomerates. Determination of the LMW6 subunit localization within the cell via **immunofluorescence** microscopy revealed indeed dense particles in the cytoplasm in varying numbers, which could represent intracellular vesicle (e.g. endoplasmatic reticulum, Golgi) or cytoplasmic agglomerations like inclusion bodies.

Because no improvements of WSP partition could be obtained by the several approaches (application of detergents, reducing agents and thermoseparating ATPS), the **cell disruption time** was further investigated. This investigation revealed that partially top phase partitioned LMW6 subunit was free before cell disruption was actually performed. Bottom phase detected LMW6 subunit increased up to 5 minutes of cell disruption, but prolonged cell disruption exceeding 5 minutes did not further increase the amount of detectable LMW6 subunit. The chosen system was not able to enrich the specific protein, which was released by cell disruption. This observation indicates different states of the LMW6 subunit measured before and after cell disruption, because partition coefficient of the LMW6 subunit differed. Since it was not possible to receive a sufficient WSP recovery with the above described ATPS, a new strategy was employed, which should lead to a system that distribute the intracellular content into PEG-rich top phase in a first system and recover WSP from host cell

contaminants in a second system. **Drastic conditions** (K_3PO_4 pH13 and PEG 300) led to a system (15% (w/w) PEG 300 / 15% (w/w) K_3PO_4 pH13, 50% lysate), which showed accelerated phase partitioning and a complete top phase directed partition of WSPs. Interestingly, only empty cell walls could be microscopically found in the bottom phase. Nucleic acid partition was directed to the top phase. This observation was expected, because it was reported that DNA was partitioned into the top phase if PEG MW is lower than 400 Da (Ribeiro et al., 2002). The partition of insoluble particles is comparable to the extraction of chloroplasts (Westrin et al., 1976), inclusion bodies (Walker and Lyddiatt, 1998) or virus like particles (Andrews et al., 1995). The separation of small bioparticles by interfacial partition was also earlier described (Jauregi et al., 2002).

Although second systems were identified, which resulted in clear PEG-rich top phases and protein enriched bottom phases, the purification and specific protein concentration was not increased. Other, more successful systems could not be identified. In some publications the second system for the recovery of the desired protein was not successful performed. Here precipitation, dialysis or filtration (Conrad and Lee, 1998) was employed to recover the product from PEG-phase. Because of the high molecular weight of WSPs cross flow filtration could be performed (Guan et al., 1994).

The specific separation of different fractions of the yeast lysate and the easy application of ATPS without cost intensive equipment could be demonstrated. Unfortunately, the aggregated state of intracellular WSPs did not allow a phase separation as described for soluble proteins. Particle partition could not be sufficiently performed to purify WSPs, because it was strongly contaminated by host cell proteins. Further improvements of the partition could be obtained, if the solubility of WSPs could be increased or agglomeration could be prevented. The efficient secretion of recombinant proteins without unwanted glycosylation might be a task to improve the applicability of ATPSs. Furthermore, it would decrease downstream effort by eliminating cell disruption step.

5 Conclusion and outlook

Several wheat storage proteins subunits were successfully expressed in the yeast *S. cerevisiae*. High amounts of recombinant proteins could be obtained by the optimization of cultivation conditions, by the application of high cell density fermentation and the improved purification. Although great improvements were obtained, process techniques were limited by the properties of the host strains. Auxotrophic marker prohibits the use of complex media, which could further improve growth and recombinant protein expression. Also the growth on glycerol as sole carbon source will be possible, which was shown to simplify high cell density

fermentation. The intracellular localization of proteins limits the product yield and hinders purification. Because intracellular volume is limited and the intracellular accumulation of recombinant proteins may cause cell stress, higher yield of WSPs could be obtained, if they were secreted into the medium. Also the purification is hindered by the intracellular localization. Efficient secretion would eliminate cell disruption, which is the main reason for host cell protein contamination. The application of aqueous two-phase systems would become more effective, even if particle partition would be performed. If extracellular agglomeration would occur, more simplified purification would be obtained by simple precipitation and centrifugation steps.

Because of the chosen WSP subunits, it was assumed that no celiac toxicity would be caused, what recently published was not the case (Dewar et al., 2006). That gliadins and low molecular weight glutenins show immunoreactive properties in celiac patients was described earlier (Rocher et al., 1995; Wieser, 1995). It can be supposed, that all chosen WSPs contains epitopes that trigger celiac toxicity. Therefore, for the production of wheat based food without celiac toxicity new ways have to be gone:

- Selection of non-toxic varieties of wheat (Londei et al., 2005; Spaenij-Dekking et al., 2005)
- Detoxification of gluten by targeted mutagenesis at genetic level (Vader et al., 2003)
- Substitution of wheat storage proteins by polymers with similar rheology (e.g. polyglycanes)
- Separated cultivation and processing of cereals without celiac toxicity (oats and teff)
- Provision of nutrients with proteases that cleaves peptides with celiac toxicity (Siegel et al., 2006; Stepniak et al., 2006)
- Addition of protease secreting *lactobacilli* to sourdough bread made from wheat for increasing tolerance thereof (Di Cagno et al., 2004)
- Application of zonulin inhibitor, which decreases gut permeability caused by WSPs (Fasano et al., 2000; Drago et al., 2006)

Although the listed ways are thinkable, several disadvantages might prevent the future applicability. The selection of non-toxic varieties will be hardly achieved. The long time results of addition of proteases or even microorganisms are not investigated so far. Nevertheless, the increase of the quality of life of celiac patients is the major aim of all listed approaches.

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IV. APPENDIX

Aqueous two phase systems – a small review

More than a century ago, aqueous two-phase systems were firstly described by [Beijerinck \(1896\)](#) in Delft in the Netherlands. Good six decades later, Albertsson realized the possible value for biomolecule isolation ([Albertsson, 1961](#)) and in 1986 he summarized his work in a book ([Albertsson, 1986](#)). Since the discovery of ATPSs, plant and animal cells, microorganisms, fungi and its spores, viruses, chloroplasts, mitochondrion, membrane vesicles, proteins and nucleic acids were purified with the help of ATPS. In the last decades the knowledge of ATPS has increased dramatically. Despite this fact, separation of a specific product cannot be predicted so far and thus each system has to be adapted empirically. The high amount of publications can only give hints to the expectable distribution within the system.

The separation commonly takes place in two steps: At first the adjustment of equilibrium and secondly the separation of the phases. The equilibrium is caused by strong mixing of the phases and subsequent separation. The separation needs more time than with organic solvents, due to the small density differences of the phases, the viscosities and the development of bigger aggregates. The separation can be sped up by the above mentioned processes. The entire process can be shortened, if necessary disruption of cells takes place in present of all system components ([Su and Feng, 1999](#)). Phase mixing and disruption is thus achieved simultaneously. After the separation occurrence, the specific protein enriched phased is removed and further purified. The first system should remove cell debris, a majority of host cell proteins, lipids and nucleic acids. An optimal purification step would lead to a PEG-rich top phase with enrichment of the desired product. Commonly, a second system is employed to recover the product into the salt rich bottom phase. Here further purification and removal of lipids, nucleic acids and colored pigments takes place. The product-free polymer phase can thus be recycled to reduce process costs. If the second system is performed with an appropriate salt, a precipitation through concentration shift could be employed to concentrate the product and remove salt phase.

In some publications this second system was not successful or not performed. Here precipitation, dialysis or filtration ([Conrad and Lee, 1998](#)) is employed to recover the product. Because of the high molecular weight of WSPs cross flow filtration could be performed ([Guan et al., 1994](#)).

For the purification of enzymes ATPS found already numerous utilization (Fexby and Bulow, 2002; Yanagida et al., 2004; Xu et al., 2005). They are especially suitable through the high water content and the protecting extraction. The removal of product from location of synthesis is not only important for in situ synthesis of antibiotics to avoid enzymatic hydrolyses (Hernandez-Justiz et al., 1998), it is also used for bioconversion (Chang et al., 1992; Zijlstra et al., 1998) to separate product from fermentor broth to reduce reuse of product or inhibitory influence of product to culture. This is achieved through **extractive fermentation** with ATPS (Sinha et al., 2000; Sinha et al., 2000). This process design is complicated due to the limited oxygen intake rate. It is thus not applicable for HCDF. Furthermore, the phase forming components has to be exchanged by non-toxic polymers as poly (ethyleneimine) (PEI) or hydroxyethylcellulose (HEC), because PEG at low molecular weight (Kuboi et al., 1995) and high salt concentrations are inhibitory to growth. Despite this fact, several ATPS were either performed in polymer-polymer (Alam et al., 1989; Oliveira et al., 2004) or polymer-salt systems (Zijlstra et al., 1998; Kulkarni et al., 1999).

Aqueous two-phase systems (ATPS) are based on the **incompatibility** of polymers or mixtures of polymer and salt. Above a certain concentration of phase forming components or with decreasing solubility conditions the single-phase system converts into a two-phase system. Substances are differently distributed when added to ATPSs, even if they are components of a complex mixture. Small molecules are distributed in this case more uniformly, whereas macromolecules separate very different and particle frequently one-sided. The distribution is dependent on a variety of parameters which makes the prediction of a distribution difficult. However, this can be also useful because by modifications of individual parameters the entire complex distribution can be changed and such preferred distribution can be achieved.

The phase diagram (PD)

The PD marks the “working area” for a specific ATPS at certain parameters, as pH, temperature and salt concentrations. It gives the composition of top and bottom phases. The binodal divides the phase diagram into two areas; above the binodal concentrations of phase forming substances are achieved, which causes a separation. Below the binodal the mixtures are homogeneous. Every point above the binodal corresponds to an ATPS with different compositions of phase forming substances. The line joining points representing the top and bottom composition is called the tie-line. It represents a straight line that combines two points of the binodal on which the concentration of phase forming substances in the top- and bottom-

phase are equal and can be determined. The ratio of the tie line sections above and below the ATPS gives information about the volume ratio of top to bottom phase.

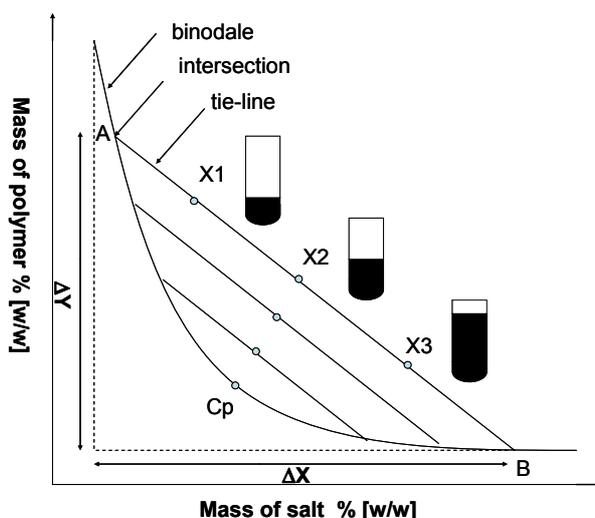


Figure 38: ATPS: Scheme of a phase diagram

X1-3 represent the composition of 3 ATPSs with different volume ratio, the composition of the top and bottom phase are reflected through the points A and B, the critical point (C_p) is determined by the extra polarization of the centers of the tie line.

The volume ratio and the concentration ratio of top and bottom phase depend in this case directly on the concentrations of the used components. The critical point (C_p , Figure 38) marks a system, where composition and volume of the two phases become equal.

Parameters influencing phase partition

The phase formation can be explained by different cross correlating parameters. Main parameters for separation are: the molecular weight of the polymer (Albertsson et al., 1987; Forciniti et al., 1991); kind of salt (Hart et al., 1995); isoelectric point of the product (Akerlund et al., 1979); overall pH of the system (Gulyaeva et al., 2003); the charge of product (Fan et al., 1998; Andrews et al., 2005); the charge of phase components (Sivars et al., 1996; Jonsson and Johansson, 2003); hydrophobicity of product (Tubio et al., 2004); hydrophobicity of phase components (Rogers and Zhang, 1996) and affinity structures (Antov et al., 2006; Everberg et al., 2006).

An important parameter is the free volume in the different phases. Figure 39 shows the dependence of the free volume on phase component concentration.

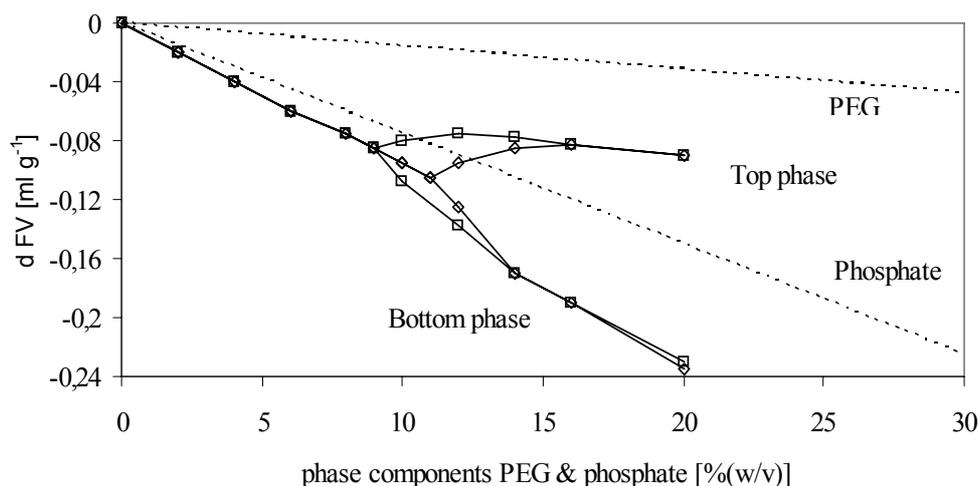


Figure 39: ATPS: Change in free volume with total composition of PEG/phosphate
Squares and diamonds denote PEG3350 and PEG 1000 ATPS, respectively, adapted (Huddleston et al., 1991)

At low concentrations of phase forming compounds the free volume in mixed systems is equal to the sum of single compounds. Above a certain concentration solubility of PEG reaches saturation and phase separation occurs. Phase separation occurs, because two different and incompatible water structures surround the polymers (Huddleston et al., 1991). The degree of substitution of water-cation hydration by PEG oxygen is responsible for the quantitative phase separation. Free volume decreases rapidly with increasing concentration in the bottom phase, whereas top phase free volume remains almost constant. Concentration of the salt in the bottom phase depends only on the overall salt concentration and not on the PEG MW. Therefore, excluded volume of PEG varies from salt to salt and from protein to protein (Huddleston et al., 1996). The addition of monovalent cations up to 1.2M increases PEG directed partition by increasing free volume due to water structure breaking (Farruggia et al., 2004).

Another important parameter that is influenced by the phase component concentration is the tie line length (TLL). Increasing TLL is accompanied by increased salt concentration in the bottom phase, what leads to salting out of proteins, when solubility limit is reached.

Kinetic behavior and time of separation depends on which of the phase is continuous in dispersion (Merchuk et al., 1998; Asenjo et al., 2002). Continuous phase is not determined by composition alone, but also by the fluid dynamics, gentle agitation created a bottom-continuously, while strong agitation produces top-continuously suspensions. Therefore, two inversion points exist in between phase continuity depends on fluid dynamics (Merchuk et al., 1998).

Addition of neutral salt to an established PEG-salt system is expected to eliminate the electrostatic effects in favor of hydrophobic effects (Huddleston et al., 1991). Several models are known for the theory of phase formation in ATPS. A review was published some years ago (Cabezas, 1996).

Different systems are known so far. They can be divided into three groups. The first and most investigated are the **polymer-polymer systems**. Most prominent system is the PEG/dextran system. It is well examined and often used in small scale and it was performed for the extraction of wheat storage proteins (Truust and Johansson, 1996). The limit for large scale is the high price for dextran. This is circumvented by the use of other polymers replacing dextran. Poly (vinyl alcohol) (PVA) (Kokkoris et al., 1988; Wu et al., 2001), hydroxypropyl starch (Sturesson et al., 1990; Venancio et al., 1993; Almeida et al., 1998) and its purified form Reppal PES 100, starch modified by acrylamid (Pietruszka et al., 2000), ethylene and propylene oxide copolymer (EOPO)-maltodextrin (Bolognese et al., 2005), pH-responsive copolymer (Waziri et al., 2004), cashew-nut tree gum (Oliveira et al., 2002; Oliveira et al., 2004), maltodextrin (Alves et al., 2000), PEG-polyethylenimin (PEI) conjugates and EOPO-PEI-conjugates (Planas et al., 1999) and combinations hydroxypropyl cellulose/PEG-co-PPG in saline buffer (Skuse et al., 1992) have been investigated. Comparison between hydroxypropyl starch and its purified from Reppal PES 100 showed similarities and only moderate differences in recovery yield (Venancio et al., 1996). In such case the cheaper hydroxypropyl starch has to be preferred.

Second and also more preferred systems are the **polymer-salt systems**. Polymer salt systems are preferred to polymer-polymer systems as a result of lower viscosity, lower cost of chemicals and shorter separation time. Here usually PEG is combined with polybasic salts to achieve phase separation. PEG-phosphate (Benavides and Rito-Palomares, 2004) is the most examined, but also PEG-sulfate (Rostami and Alamshahi, 2002) and PEG-citrate (Oliveira et al., 2003; Zhi et al., 2004) are successfully applied. Industrial utilization mainly bases on PEG-salt system and only few on the more expensive PEG/dextran or PEG/rawdextran system. For the same reason and because of PEG-phosphate system was shown to be suitable for the partition of yeast proteins, some years ago (Flanagan et al., 1991), the system should be suitable to isolate WSPs from yeast lysate. PEG is a polymer composed of repeating ethylene residues linked by an ether bond. The other oxygen site can bind two water molecules. PEGs state of aggregation depends on chain length. PEG with MW of 300Da is liquid, 600Da is liquid above 30°C, and 1kDa appears mucinous, whereas higher chain length appears solid.

Effectiveness of a salt is more affected by **the anion** than the cation (Huddleston et al., 1991) and at higher valences. PO_4^{3-} and SO_4^{2-} are very effective with PEG. The Galvanic-type interfacial electrostatic potential difference $\Delta\psi$ of PEG-sulfate and PEG-phosphate systems is positive, so that it favors partitioning of net negatively charged proteins into the PEG-rich top phase (Johansson et al., 1998).

The disadvantage in large scale is the high concentrated salt containing waste. This is circumvented by the use of volatile salt as ammonium carbamate, which shows also phase forming properties (van Berlo et al., 2000; van Berlo et al., 2000). Ammonium bicarbonate is not suitable because of its low solubility (van Berlo et al., 1998). Polymer-salt-systems normally build a high polymer, low salt containing top phase and low polymer, high salt containing bottom phase.

The last group is the **detergent based ATPSs**. The possibility to achieve phase separation by temperature change enables the use of only one system component and gives the opportunity to recycle the polymer. Thermoseparating polymers are the non-ionic Triton series (polyethylene alkyl phenols) as Triton X114 (Ramelmeier et al., 1991; Nunez-Delicado et al., 1996), the Tween series (polyoxyethylene sorbitol ethers), the non-ionic alkyl polyoxyethylene ethers (C(m)EO(n), as C(11)EO(2) (Linder et al., 2001), random copolymer of ethylene oxide and propylene oxide (EOPO) (Kepka et al., 2004; Li and Peebles, 2004), dodecylmaltoside (DDM) (Everberg et al., 2004), dodecyltriethylammonium bromide and SDS (Xiao et al., 2000). Even the technical non-ionic surfactant Agrimul NRE 1205 (Selber et al., 2001) and were used and investigated.

The **combination of such systems** is also possible and already performed. The use of two detergents gives the opportunity to recycle both polymers after the first phase separation by thermoseparation (Persson et al., 2000; Everberg et al., 2004). The use of detergent-salt systems, as Triton X100- K_2HPO_4 (Qin and Zhao, 2003) or TX-100-phosphate (Stalberg and Larsson, 2001) and detergent-polymer systems (Sivars and Tjerneld, 2000) profit from this advantage, too.

The adaptation of a system to the certain product can be performed by different approaches. The use of **affinity ligands** is one of the most specific and most applied. Ligands can be **freely added** to the systems as reactive triazine dyes (Xu et al., 2003), Eudragit S-100 (copolymer of methacrylic acid and methyl methacrylate) and alginate in PEG-salt systems (Teotia et al., 2001), free starch as bioligand for glucoamylase (de Gouveia and Kilikian, 2000) and ligand carrier poly-N-isopropylacrylamid copolymerized with itaconic anhydride modified monoclonal antibodies (Kumar et al., 2001). Eudragit S-100 is a polymer, whose

solubility can be changed by pH change. Therefore, it is used for affinity precipitation (Guoqiang et al., 1994).

Polymers as polyvinylpyrrolidone (PVP40) **complexing** azo dyes and inorganic ions can be used for dye or metal affinity partition e.g. in a PVP40- Reppal PES 100 system (Fernandes et al., 2002). The most prominent affinity partition is performed with the **covalent attachment** of affinity ligands to the polymer. In that way fatty acids (Johansson and Shanbhag, 1984), triazine dyes (Birkenmeier et al., 1984), Cibacron Blue F3G-A (Tejedor et al., 1992), iminodiacetic acid (IDA)-Cu(II) for metal affinity (da Silva and Franco, 2000; Sivars et al., 2000), avidin for biotinylated products (Nishimura et al., 1995) as liposomes (Ekblad et al., 1998), hydrophobic modification to EOPO (Persson et al., 1999), peptides as affinity bioligand for vancomycin recovery (Singh and Clark, 1994), wheat-germ agglutinin linked to dextran for plasma membrane recovery (Persson and Jergil, 1992), benzoyl group to dextran (Lu et al., 1991), textile dyes to PVP (Giuliano, 1991) and albumin to PEG coupling at mild conditions (Delgado et al., 1990)

But also the recombinant **products can be labeled by ligands**, as done with hydrophobic metal binding fusion tags for metal affinity partition (Bernaudat and Bulow, 2005), charged tags (Bandmann et al., 2000), tryptophan rich peptide fusion (Carlsson et al., 1996) and other peptides (Hassinen et al., 1994; Collen et al., 2001) or changed for enhanced partition property by substitution of surface amino acids (Berggren et al., 2000).

Phase separation can be performed by gravitation or is enhanced by the use of centrifuges or separators that increases gravity. The H/D-ratio of separation device is decisive for separation time (Solano-Castillo and Rito-Palomares, 2000) due to higher cross section area. For the proper determination of volume ratio this is limited.

The enhancement of demixing is also achieved by applying microwave (Nagaraj et al., 2003), acoustic (Srinivas et al., 2000; Srinivas et al., 2001; Nagaraj et al., 2002), electric (Raghavarao et al., 1998) or magnetic field. Magnetic field obviously functions, if iron particles are added, especial if 1-micron iron particles are used (Flygare et al., 1990), or if a modified polymer as dextran-ferrofluid is used (Wikstrom et al., 1987). Drop coalescencers (ceramic rings, PTFE and glass) can also speed up phase separation (Ban et al., 2001). A general overview of aqueous two-phase systems was already given some years ago (Hatti-Kaul, 2001).

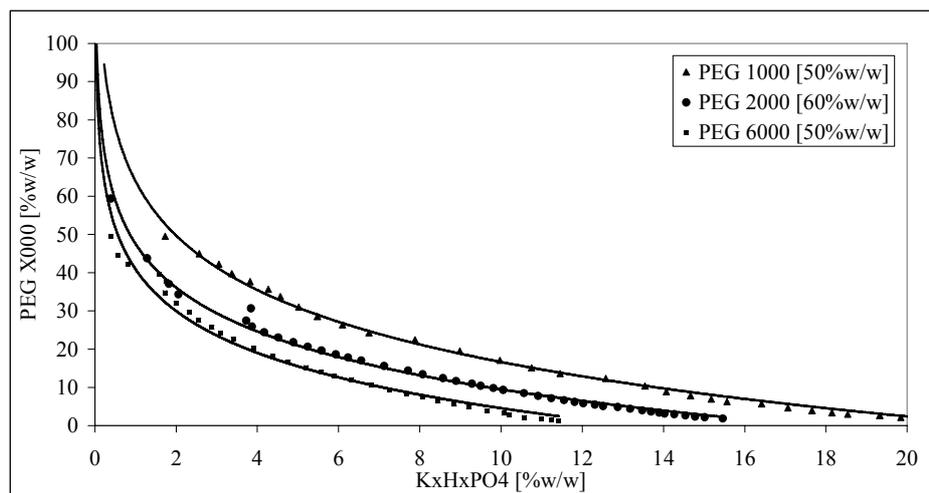


Figure 40: ATPS: Determination of binodals using PEG with different molecular weight

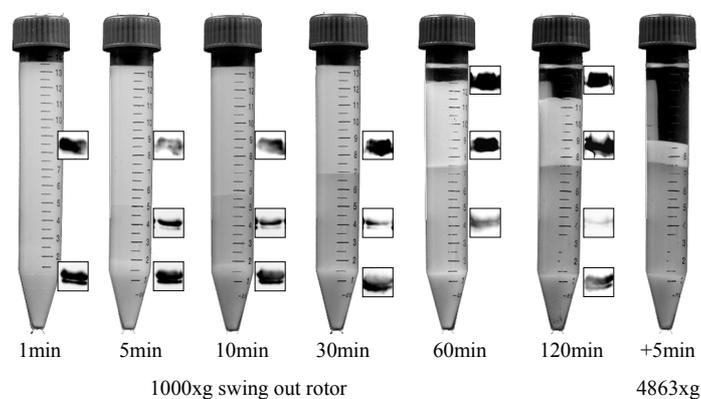


Figure 41: ATPS: Time series of phase formation during centrifugation
Boxes indicate the western blot of a phase aliquot

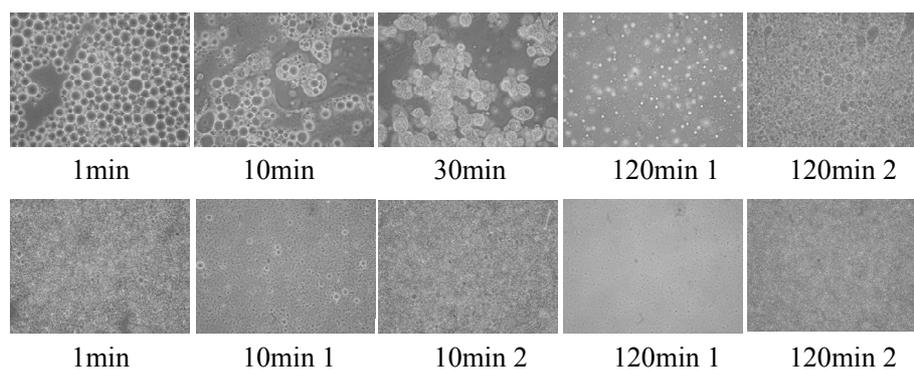


Figure 42: ATPS: Microscopic pictures of the different phases during centrifugation
Upper row shows top-phase and lower bottom phase development; TP 120min as well as BP 10 and 120min split in two bands

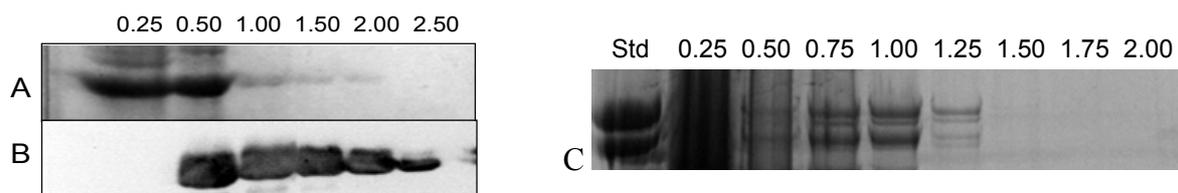


Figure 43: ATPS: Influence of 2-propanol/yeast-lysate ratio on precipitation of diff. WSP from yeast (A) ponceau S stained membrane (B) western blot detection A; lane 1-6: 0.25 - 2.50 2-propanol/LMW6-containing-lysate-ratio supernatant; (C) coomassie stained excerpt of a gel, lane 1: standard; lane 2-9: 0.25 - 2.00 100% 2-propanol/HMW-containing-lysate ratio supernatant

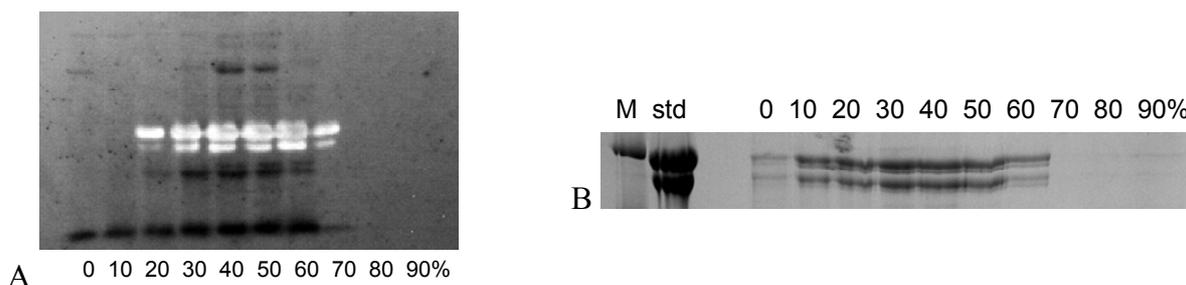


Figure 44: ATPS: Influence of 2-propanol concentration on extraction of diff. WSP from yeast (A) Ponceau S stained membrane (dark) with overlay of western blot detection (light) of LMW6-containing lysate of different 2-propanol containing extraction buffer; (B) coomassie stained gel with extracts of HMW-containing lysate of different 2-propanol containing extraction buffers

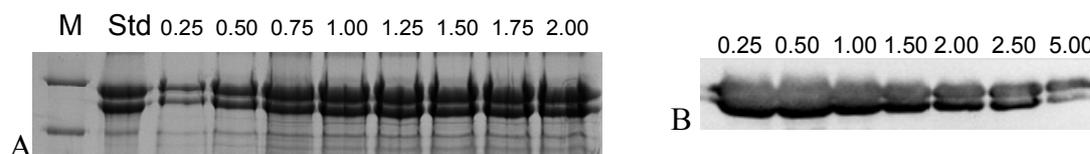


Figure 45: ATPS: Influence of buffer/yeast-lysate-ratio on WSP extraction from yeast (A) Coomassie stained gel with HMW-subunits after extraction with different extraction-buffer/lysate-ratio; (B) western blot detection of LMW6-subunit after extraction with different extraction-buffer/lysate-ratio (intensity was multiplied by volume)

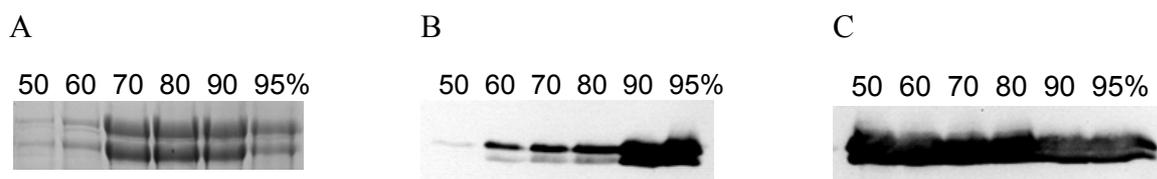


Figure 46: ATPS: Influence of 2-propanol concentration on WSP extraction from yeast lysate (A) Coomassie stained gel of HMW-subunits precipitated with different 2-propanol concentrations in the mixture at 4°C, upper band HMW-1Dx5, lower band HMW-1Dy10, (B) western blot detection of LMW6-subunit precipitated with different 2-propanol concentrations in the mixture at 25°C and (C) 4°C

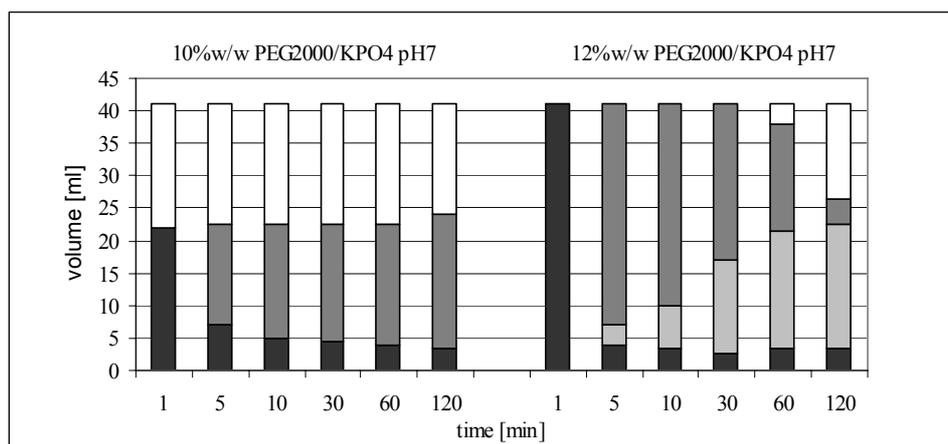


Figure 47: ATPS: Volume ratio of two selected ATPS after different times
Grey scale indicate approx. turbidity of the phases resulting from biomass load

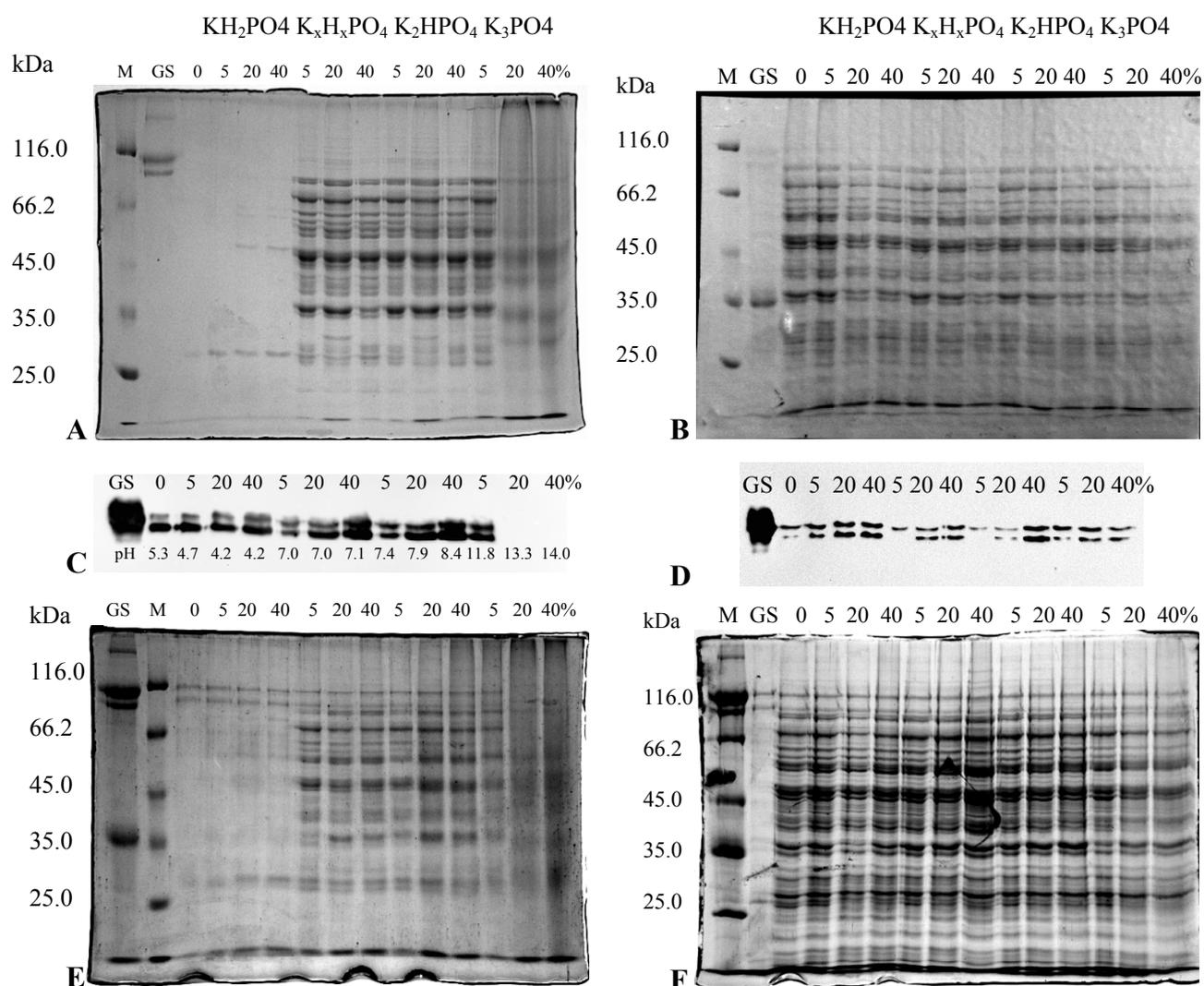


Figure 48: ATPS: Images of detected WSP in buffered and unbuffered extractions
Coomassie stained gel and ponceau S stained membrane of unbuffered (A) and buffered extraction of LMW6 (B); western blot detection of unbuffered (C) and buffered extraction (D) of LMW6; coomassie stained gel of unbuffered (E) and buffered extraction (F) of HMW.

Die vorliegende Arbeit wurde am Institut für Biotechnologie im Fachgebiet Mikrobiologie und Genetik der Technischen Universität Berlin unter der wissenschaftlichen Betreuung von Prof. Dipl.-Ing. Dr. Ulf Stahl angefertigt.

Versicherung

Hiermit versichere ich, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht.

Die Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Die Promotionsordnung wird anerkannt.

Berlin, den 29.08.2006

Falk Matthäus