7 Summary

The main objective for this work was the examination of the biotechnological power of the *L. tarentolae* expression system. Static and dynamic process parameters had to be determined for an optimal cultivation of the promastigote cell type of *L. tarentolae*, because promastigotes can produce recombinant proteins with an animal-like N-glycosylation pattern [Breitling *et al.* (2002)].

The nutrient media is the important static process parameter. Different nutrient media are now available for the cultivation of *L. tarentolae*, which support high growth rates ($\mu = 0.1\ h^{-1}$) and cell densities (6.5×10^8 - 1×10^9 cells/ml). A nutrient media, containing only hemin as animal-derived substance, could be established and evaluated with the YE-medium. The maximal specific growth rate is $0.103\ h^{-1} \pm 0.007\ h^{-1}$ in the YE-medium. It could be prepared relatively easy and cheap.

The YE-medium is a complex nutrient media, containing a mass of substances with unknown concentrations. Nevertheless, the media was successfully used for growth experiments, also under limiting glucose or hemin concentrations (chemostate), for investigations to the primary carbon source, to the iron source and to physiological parameters like temperature or pH.

L. tarentolae could be cultivated as static culture, in shaker flasks or bioreactors. The least doubling times (6.7 h in the YE-medium) offered the cells under shaked or agitated conditions. The developed media are appropriate for bioreactor cultivations. The long-term stability of growth was demonstrated for more than 50 passages as static suspension culture in the YE-medium. Thus, constant initial conditions for larger scales are provided for about half a year.

Glucose was proved as primary carbon and energy source, whose metabolism in complex media was accompanied with an acidification of the pH. The yield coefficient $Y_{X/GLC}$ was determined to 0.93 ± 0.09 gram cell dry weight per gram glucose. A glucose-limited cultivation (chemostate) was possible with complex medium, resulted in determination of the Monod-constant (K_S) to 0.046 g/l glucose. *L. tarentolae* showed a huge tolerance to glucose, because a growth inhibition occurred first at 60 g/l glucose (at 26°C cultivation temperature) and 40 g/l (at 30°C) respectively.

Hemin is the essential growth factor for the cultivation of *L. tarentolae* and an interesting control factor for a bioprocess. Therefore, the fundamental prerequisite was the establishment of an analytical method, which succeeded with an aqueous two-phase extraction in acidic chloroform. The identification of the main influencing variables to this analytical method resulted in a final standard procedure. For an exponential growth, a yield

coefficient ($Y_{X/H}$) of 834 gram cell dry weight per gram hemin could be determined by comparison to a standard plot. This yield coefficient represents all “hemin-consuming” procedures during the cultivation, because the free hemin concentration was influenced largely by the adsorption of hemin to the glass surface of the cultivation vessel. Even if only a portion of the declared hemin is used by the cells, addition of the whole hemin is necessary in order that sufficient hemin is available for the cells. The determined yield coefficient is applicable for bioprocess modelling, which was exemplified with a hemin-limiting chemostate. The cells of the cultivation followed the hemin-limitation of the process. This was surprising, because details to a hemin accumulation of the cells existed. The cells could continue growth for approximately 3 generations under hemin deprivation. A Monod-constant for the substance hemin (K_H) could be calculated to 31 µg/l. This is an important factor for future investigations. Hemin is the only substance of animal origin (bovine or pork), but currently it could not be replaced by an alternative substance or a commercially available, synthetic hemin preparation.

During these studies, an optimal growth of *L. tarentolae* could be determined at a cultivation temperature of 30°C. This was approved in the shaker flask and in the bioreactor. This result was very astonishing, because promastigotes of the *Leishmania* species were normally cultivated at 22 – 28°C, but mainly at 26°C. In general, higher temperatures induce the transformation to the amastigote cell type. The increase of the cultivation temperature from 26°C to 30°C resulted in a rising of the specific growth rate at about 30%. The doubling time was reduced from 6.9 h to 5.4 h. This could be a considerable saving in time for a production process.

As a basic principle, the optimal pH-range for the cultivation is the neutral one (pH 6.9 – 7.6). For cultivations in shaker flasks, the use of a pH-buffer system and well-timed passages into new media are recommended. The cells tolerate different buffer systems like citric acid, potassium- or sodium phosphate or Tris/HCl. For bioreactor cultivations, the regulation of the pH is advisable. Growth inhibitions occurred out of the optimal pH-range. At pH > 7.6 cell degenerations started and by reduction to pH 5, an incomplete transformation to the amastigote cell type appeared.

Drastic variations of the extra cellular environment or the metabolism are represented in morphological changes of the cells. This is an important conclusion for characterization of the growth. Degeneration of the cells occurred at pH > 7.6 and in old cultures. If cells grew at high growth rates, e.g. engage / wash out of the chemostate or exponential growth in a shaker flask, then the length of the flagellum was less the length of the cell body. Partially, the flagellum was extremely shortened or degenerated. The cell body was always thick and drop-like. In contrast, clearly extended flagellates, about twice the length of the cell body,

were shown by cells growing under limited conditions (steady state of the chemostate). The cell body was much thinner, but also drop-like. The glucose-limited cultivation showed a dependency between morphological changes and dilution rate, because the cells were much thinner at a small dilution rate than at a larger one. After the switch in metabolism, the cell appeared very thin with longer flagellates. No morphological changes were recognized during the variation of the cultivation temperature.

Furthermore, different fedbatch-strategies for high cell densities during growth on glucose were designed. It is recommended to hold glucose and hemin unlimited and to feed a concentrate of the nutrient media. With this strategy a maximal cell density of 1.8×10^9 cells/ml (X_{max} = 14.5 g/l CDW) could be achieved in the YE-medium and under metabolism of glucose. This is a clearly higher cell density compared to cell densities of previous methods and media (shaker flask with YE-medium: X_{exp} = 3.4×10^8 cells/ml). In contrast to literature values [Simpson *et al.* (1996)] meaning these 4.5 higher cell densities.

A stirrer range of 100 rpm to 400 rpm is advisable for a bioprocess (by use of 2-blade turbines). Lyses of some cells started at 400 – 500 rpm. Advantageously is the regulation of the pH by using caustic potash or caustic soda, the use of silicone oil to reduce foam and the utilization of PEG-hemin, which has an anti-foam effect through the PEG1000-portion.

During the development of the defined SFP-media problematic hemin precipitations occurred, which could be prevented by the use of BSA and PEG1000. PEG1000 is advantageous to BSA because of the synthetic production and the reduced formation of foam. The cell density in the defined SFP-media could be nearly doubled by addition of yeast-RNA (N_{max} = 2.8 – 3.3×10^8 cells/ml). The metabolism of the yeast-RNA and also the significant reaction of the cells to the addition of this substrate could be demonstrated. By using a fedbatch strategy, which includes the exchange of nutrient media, the process could be hold for five cell generations. Furthermore, important orientations are offered by the process parameters pH, pO_2 and CO_2.

The investigations of this work occupy that under the used experimental conditions the Monod-model is in power for the growth of *L. tarentolae*, excluding glucose inhibition. Moreover, it was clearly pointed out, that the effects of substrates are greater than the influence of temperature or pH. This inaugurates good control possibilities for μ or ν, respectively.

Basic parameters and strategies for an optimal cultivation were detected and designed by using the wild type strain. These could be utilized as orientation for the use of concrete host-vector-systems. The earned knowledge's were transferred to the expression of the model proteins hyaluronidase-1, surface antigen SAG2 of *Toxoplasma gondii* and EGFP. The

developed nutrient media supported the expression of the recombinant proteins. The host-vector-system with intracellular expression of EGFP showed nearly identical growth rates in comparison to the wild type-organism. In contrast, a degrease of μ and ν was registered for the constitutive, secretory SAG2-strain, which was independent to the used nutrient medium. The expression of the SAG2-protein was proved by detection of the fused His_6-tag in the western blotting after a one hundred-times concentration of the media supernatant. Determination of the protein concentration was not possible. Further investigations have to show the antigenicity of the recombinant SAG2. During the bioreactor cultivation, the formation of the SAG2 did not followed exponential growth. The specific SAG2-ratio decreased tendentiously. A product inhibition is assumed.

With the hyaluronidase-1, a recombinant glycoprotein has been expressed. The correct genomic integration of the *hyal-1* gene was verified. The specific Hyal-1 activity in the media supernatant was eight times higher than the reported one for insect cells [Hofinger *et al.* (2007)]. But a concrete protein band in the SDS-PAGE could not be identified, because the protein concentration was too small, also after TCA-precipitation. In the western blotting a very thin band was detected. Under consideration of the cell density and the shortened induction time, *L. tarentolae* has a higher productivity compared to insect cells. In that, the great potential of this new system is shown. The high activity values indicate a natively folded and glycosylated hyaluronidase-1.

The results of this work enlarge clearly the knowledge about the *L. tarentolae* expression system, mainly in preparation of a biotechnological utilization. The basis for future bioprocess strategies and optimizations is set with the determination of the Monod-constants for glucose and hemin, the maximal specific growth and doubling rates and also the yield coefficients for glucose and hemin. High cell densities in combination with doubling times of 5-7 h are possible with the developed nutrient media and strategies. Therewith, prerequisites for the use of the promastigotes of *L. tarentolae* for production of recombinant proteins for diagnostic or therapeutic purposes at humans are made. Because of the possibility for N-glycosylation of human proteins, *L. tarentolae* is an alternative system to mammalian cells like BHK or CHO-cells.