

## Research Paper

## Characterization of the growth behavior of *Leishmania tarentolae* – a new expression system for recombinant proteins

Claudia Fritsche<sup>1</sup>, Mandy Sitz<sup>1</sup>, Norman Weiland<sup>1</sup>, Reinhard Breitling<sup>2</sup> and Hans-Dieter Pohl<sup>1</sup><sup>1</sup> Department of Medical Engineering and Biotechnology, University of Applied Sciences Jena, Jena, Germany<sup>2</sup> Jena Bioscience GmbH, Jena, Germany

Biotechnological production of recombinant proteins for human therapy requires a cultivation of the host organism in a nutrient medium free of animal substances. Therefore, various nutrient media for the new expression system *Leishmania tarentolae* were developed and examined according to their cultivation conditions as static suspension culture and agitated culture. Investigations resulted in the development of a serum-free but hemin containing medium, based on yeast extract and buffer salts. Here we report that a high and stable specific growth rate of  $0.103 \text{ h}^{-1}$  and a maximal cell density of  $1 \times 10^9 \text{ cells ml}^{-1}$  is obtained in an alternative medium, the YE-medium. For the newly developed medium, the successful expression of enhanced green fluorescent protein and the adaptation of the cultivation from the agitated culture to the bioreactor could be shown. Furthermore, an analytical method for detection of the essential, organic iron source hemin was established. The consumption of hemin was monitored because hemin is a potentially important process parameter for bioprocess control. With knowledge of these results, an improved expression system is available as an alternative to commonly used cell cultures for the production of recombinant proteins.

**Keywords:** *Leishmania* / Nutrient media / Hemin detection / EGFP expression / Specific growth rate

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### Introduction

*Leishmania* species are protozoa of the genus *Trypanosoma* with a complex life cycle (promastigotes and amastigotes). *Leishmania tarentolae* is a parasite of the gecko *Tarentolae annularis* and has been developed as new eukaryotic expression system for the production of recombinant proteins with an animal-like N-glycosylation pattern, as shown by Breitling *et al.* for erythropoietin expression (Breitling *et al.* 2002). The system is available with constitutive or regulated transcription (Breitling *et al.* 2002, Kushnir *et al.* 2005) and can be considered as an alternative expression system

to mammalian cell cultures (Sodoyer 2004). The main advantages are the higher specific growth rate compared to mammalian cells and cultivation in low cost media.

Prior to expression studies the main growth parameters for the wild type organism (parameters such as specific cell division rate ( $\nu$ ), specific growth rate ( $\mu$ ), doubling time ( $t_D$ ), number of generations ( $k$ ), main carbon source, yield coefficient ( $Y$ ) and maximal cell densities ( $N_{\max}$ )) had to be determined. To establish the system for production of recombinant proteins for therapeutic purposes, the nutrient medium must be free of animal substances due to regulatory requirements (Sodoyer 2004).

Promastigotes are mainly cultivated in liquid media to which animal serum or blood is added, and in nutrient media of animal origin (Chang and Fish 1983). A commonly used medium is the Brain Heart Infusion

**Correspondence:** Claudia Fritsche, University of Applied Sciences Jena, Department of Medical Engineering and Biotechnology, Carl-Zeiss-Promenade 2, D-07745 Jena, Germany

**E-mail:** Claudia.Fritsche@fh-jena.de

**Tel.:** +49 36 41 / 20 56 76

**Fax:** +49 36 41 / 20 56 01

**Table 1.** Comparison of growth parameters for *Leishmania* species of suspension cultures from literature (selection). All reported data observed in static suspension culture.

| Type of medium | Author                              | Species  | Ingredients of the medium                                      | $N_{\max}$<br>$\times 10^6$<br>[cells ml <sup>-1</sup> ] | $\nu$<br>[h <sup>-1</sup> ] | $t_D$<br>[h]        | $k$<br>[-]         |
|----------------|-------------------------------------|--|--|--|-----------------------------|---------------------|--------------------|
| complex        | Meehan <i>et al.</i> (2000)]        | <i>L. tarentolae</i>   | BHI + hemin  | 200  | 0.116 <sup>a)</sup>         | ~6                  | — <sup>b)</sup>    |
|                | Ali <i>et al.</i> (1998)            | <i>L. major</i>  | peptone, casein hydrolysate, beef & yeast extract              | 24   | 0.039 <sup>a)</sup>         | 17.8 <sup>a)</sup>  | ~8 <sup>a)</sup>   |
|                | Limoncu <i>et al.</i> (1997)        | <i>L. infantum</i>   | peptone, yeast extract, tripti-                                | ~2   | 0.02 <sup>a)</sup>          | ~35 <sup>a)</sup>   | 3.7 <sup>a)</sup>  |
|                | Palomino (1982)                     | <i>L. tropica</i><br><i>L. brasiliensis</i>                        | case, bovine haemoglobin<br>peptone +10% FCS, yeast autolysate | 40   | 0.024 <sup>a)</sup>         | ~28.9 <sup>a)</sup> | 5.8 <sup>a)</sup>  |
| Semi-defined   | Ali <i>et al.</i> (1998)            | <i>L. major</i>  | M199+10% serum +2% urine                                       | 22   | 0.057 <sup>a)</sup>         | ~12.2 <sup>a)</sup> | 7.9 <sup>a)</sup>  |
|                | Limoncu <i>et al.</i> (1997)        | <i>L. infantum</i><br><i>L. tropica</i>                            | RPMI 1640 +10% FCS   | 21   | 0.025 <sup>a)</sup>         | 27.7 <sup>a)</sup>  | 7.7 <sup>a)</sup>  |
|                | McCarthy-Burke <i>et al.</i> (1991) | <i>L. donovani</i>   | M199 + HEPES, folic acid, hemin, eagles vitamins               | 40   | 0.077                       | 8.9                 | ~6.3 <sup>a)</sup> |
| Defined        | O'Daly <i>et al.</i> (1988)         | <i>L. donovani</i><br><i>L. brasiliensis</i><br><i>L. mexicana</i> | amino acids, nucleotide, vitamins, salts, hemin                | ~70  | 0.03 <sup>a)</sup>          | 23.1 <sup>a)</sup>  | 6.6 <sup>a)</sup>  |
|                | Melo <i>et al.</i> (1985)           | <i>L. tarentolae</i>   | amino acids, hemin vitamins, salts                             | 35   | 0.008 <sup>a)</sup>         | 86.6 <sup>a)</sup>  | 2.5 <sup>a)</sup>  |
|                | Merlen <i>et al.</i> (1999)         | <i>L. donovani</i><br><i>L. brasiliensis</i><br><i>L. mexicana</i> | amino acids, salts, vitamins, hemin, nucleotides               | ~79  | 0.023 <sup>a)</sup>         | ~30 <sup>a)</sup>   | 7.3 <sup>a)</sup>  |
|                | Trager (1957)                       | <i>L. tarentolae</i>   | amino acids, salts, vitamins, hemin, purine, pyrimidine        | 50   | — <sup>b)</sup>             | — <sup>b)</sup>     | — <sup>b)</sup>    |

<sup>a)</sup> – calculation from literature data; <sup>b)</sup> – not specified.

(BHI), partially supplemented with serum. This medium exhibits a risk for contamination of the recombinant product e.g. with viruses or with prion proteins responsible for bovine spongiform encephalopathy (BSE; Mad Cow's disease) (Robb 1975, Yamamoto and Akama 1969). Various other media have been described (Chang and Fish 1983, Schuster and Sullivan 2002). These are summarized in Table 1. BHI-medium only allowed to obtain high cell densities of *Leishmania* species. In many publications it is emphasized, that not all *Leishmania* species and strains grow in the reported synthetic media, because they differ in their nutritional requirements (O'Daly and Rodriguez 1988, Melo *et al.* 1985, Merlen *et al.* 1999). Therefore, transfer of results from one species to another is rarely successful.

Summarizing the results from the literature, *L. tarentolae* cannot currently be cultivated to cell densities  $>2 \times 10^8$  cells ml<sup>-1</sup> with a high specific growth rate in serum-free and animal substances-free medium. Therefore, we developed an alternative medium, serum-free and containing only hemin as substance of animal origin. Hemin is essential for growth and has been added to culture medium, e.g. by Pal and Joshi-Purandare (2001), who were able to demonstrate a dose-dependent effect of hemin on protein synthesis and cell pro-

liferation in *L. donovani*. Here we have established an analytical method to determine consumption of hemin as a potentially important process parameter.

We were able to demonstrate the application of that alternative medium for expression of enhanced green fluorescent protein (EGFP) and we were also able to adapt agitated cultures to bioreactor cultivation.

## Materials and methods

### Strain and cultivation conditions

The *L. tarentolae* laboratory strain p10 (Jena Bioscience, Germany) was maintained at 26 °C as static suspension culture in 25 cm<sup>2</sup> plastic cell culture flasks filled with 10 ml nutrient broth and diluted into new medium every 2–3 days. For experiments with agitated cultures, the cells were cultivated in 250 ml shaker flasks with four baffles filled with 50 ml nutrient broth at 26 °C and 140 rpm in a shaker water bath (Julabo SW20, Julabo Labortechnik, Germany). Inoculation was done from a late logarithmic phase growing agitated pre-culture containing the examined medium, which was previously inoculated from a static suspension culture. If necessary, the inoculum was centrifuged (2000 × g,

**Table 2.** Components and preparation of nutrient media used for cultivation of *L. tarentolae*; all ingredients are dissolved in aqua dest. Autoclaving was done at 121 °C for 20 min.

| Medium          | Solution   | Ingredients                     |                        | Preparation                        |
|-----------------|------------|---------------------------------|------------------------|------------------------------------|
| LEXSY Broth BHI |            | Difco Brain Heart Infusion      | 37.0 g l <sup>-1</sup> | dissolved and autoclaved           |
| TSB             |            | BBL™ Trypticase™ Soy Broth      | 15.0 g l <sup>-1</sup> | dissolved and autoclaved           |
|                 |            | Bacto™ Yeast Extract            | 20.0 g l <sup>-1</sup> |                                    |
| TB              | Solution A | Bacto™ Tryptone                 | 12.0 g l <sup>-1</sup> | dissolved in 900 ml and autoclaved |
|                 |            | Bacto™ Yeast Extract            | 24.0 g l <sup>-1</sup> |                                    |
|                 | Solution B | K <sub>2</sub> HPO <sub>4</sub> | 12.5 g l <sup>-1</sup> | dissolved in 100 ml and autoclaved |
|                 |            | KH <sub>2</sub> PO <sub>4</sub> | 2.3 g l <sup>-1</sup>  | combine solution A and B to 1 l    |
| YE              | Solution C | Bacto™ Yeast Extract            | 24.0 g l <sup>-1</sup> | dissolved in 900 ml and autoclaved |
|                 | Solution D | K <sub>2</sub> HPO <sub>4</sub> | 12.5 g l <sup>-1</sup> | dissolved in 100 ml and autoclaved |
|                 |            | KH <sub>2</sub> PO <sub>4</sub> | 2.3 g l <sup>-1</sup>  | combine solution C and D to 1 l    |

20 °C, 10 min) to reduce volume and the pellet was used for inoculation. Minimal cell density was approximately  $2 \times 10^7$  cells ml<sup>-1</sup>.

#### Preparation of nutrient media

The media used are listed in Table 2. Ingredients were obtained from Becton Dickinson, USA. Salts for buffer preparation were of analytical grade and from Merck, Germany. Prior to inoculation glucose was added to a final concentration of 2–3 g l<sup>-1</sup> from a sterile stock solution. Penicillin-Streptomycin (Invitrogen, USA) was supplemented to 50 unit's ml<sup>-1</sup> to avoid bacterial contamination. Hemin (Sigma-Aldrich, Germany) was added to a final concentration of 5 mg l<sup>-1</sup> from a sterile stock solution of 2.5 g l<sup>-1</sup> hemin in 50% triethanolamine (Sigma, USA).

#### Determination of growth

Growth was monitored by measuring cell density using a cell counter (Coulter Z2, Coulter Electronics, USA). Optical density was determined at 600 nm with the spectrometer Spectronic 20 Genesys (Spectronic Instruments, USA). Glucose was measured by the glucometer ECA PD10 (Prüfgerätewerk Medingen, Germany). PH was measured externally with the pH Meter 526 (WTW, Germany). Pictures were taken by the Canon Power Shot G5 camera under phase contrast 2 in a light microscope (Carl Zeiss, Germany).

#### Bioreactor cultivation

*L. tarentolae* was cultivated in a 2 l stirred tank bioreactor (Biostat MD, B. Braun, Germany) at 26 °C, airflow 1 VVM and rotation speed 100–300 rpm. For reduction of shear stress, 2-blade turbines were used. PH was uncontrolled. YE-medium was prepared according to Table 2 and supplemented with 3 g l<sup>-1</sup> glucose, 50 unit's ml<sup>-1</sup> penicillin-streptomycin and 1 mg l<sup>-1</sup> hemin. Growth and glucose consumption was detected as described

previously. Cells for inoculation originated from a three days old static suspension culture (10 ml), scaled-up stepwise from 1 × 50 ml to 3 × 100 ml over two days to provide sufficient cell concentrations in the bioreactor.

#### EGFP-expression studies

For expression studies, the recombinant strain *L. tarentolae* p10::F9Begfp1.4dBsat#12 (Jena Bioscience, Germany) with the gene for EGFP (Enhanced Green Fluorescent Protein), chromosomal *ssu* integrated, was cultivated in the various media as described previously and additionally supplemented with 100 mg l<sup>-1</sup> Nourseothricin (Jena Bioscience, Germany). During cultivation, 1 ml samples were taken, centrifuged (2000 × g, 20 °C, 10 min) and washed with 0.9% NaCl solution. Pellet was resuspended in buffer (20 mM HEPES, 5 mM EDTA, 2 mM DTT) and disintegrated by sonification (application of energy ~ 400 Ws) (UP400S, Dr. Hielscher, Germany). Cell debris were removed by centrifugation (6000 × g, 4 °C, 5 min) and analyzed by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions according to the method of Laemmli (1970) with 12.5% polyacrylamide gels. EGFP-expression was examined in agitated culture.

#### Determination of hemin consumption

The determination of the hemin content was conducted with an aqueous 2-phase-system according to the method of Lombardo *et al.* (2005). Chemicals used were either from Carl Roth, Germany, or from Merck, Germany. Sample preparation was done in 15 ml Rotilabo® centrifuge tubes (Carl Roth, Germany), filled with 4 ml sample and mixed with 2 ml 50 mM glycine-HCl buffer, pH 2. If hemin concentration had to be determined in the YE-medium, 2 ml DMSO was added. After mixing, the pH was adjusted to pH 2 with 25% or 10% HCl solution, followed by addition of 200 µl of 5 M NaCl. After vortexing, 2 ml chloroform was added and mixed vig-

ously three times for 5 sec for optimal distribution of hemin in the organic phase. Phase separation was achieved by centrifugation ( $2000 \times g$ ,  $20^\circ\text{C}$  and 5 min). The bottom phase (chloroform) was transferred into a 1 ml quartz cuvette and the absorption was recorded in a microplate reader (Lambda Scan 200, BIO-TEC INSTRUMENTS, USA). Quantification of the hemin content was done by estimation of the peak area (from 340–450 nm) including base line neutralization.

The standard curve was determined with a  $5 \text{ mg l}^{-1}$  hemin solution (prepared with 0.5% triethanolamine) and diluted stepwise with aqua dest. Complete dissolution of hemin was achieved by exposing the solution for 1 min to an ultrasonic bath with a power output of 50%. Consumption of hemin was measured during growth in YE-medium in an agitated culture as described previously.

### Basic equations for the evaluation of the results

The growth of *L. tarentolae* was analyzed by calculation of characteristic values according to the equations 1 and 2. The variables are the cell density ( $N$ ) in  $\text{cells ml}^{-1}$ , the specific cell division rate ( $\nu$ ) in  $\text{h}^{-1}$ , cell dry weight ( $x$ ) in  $\text{g l}^{-1}$ , calculated from OD by a correlation factor, and the specific growth rate ( $\mu$ ) in  $\text{h}^{-1}$ .

$$\frac{dN}{dt} = \nu N \quad (1)$$

$$\frac{dx}{dt} = \mu x \quad (2)$$

Furthermore, yield coefficients were determined according to equation 3 and 4, where  $\Delta N$  is the produced cell density and  $\Delta S$  the consumed glucose concentration in a defined time interval and volume. Similarly,  $\Delta x$  is the produced cell dry weight and  $\Delta H$  the consumed hemin concentration in a defined time interval and volume.

$$Y_{N/S} = \frac{\Delta N}{\Delta S} \quad (3)$$

$$Y_{x/H} = \frac{\Delta x}{\Delta H} \quad (4)$$

## Results

### Growth kinetics of static and agitated cultures in various nutrient media

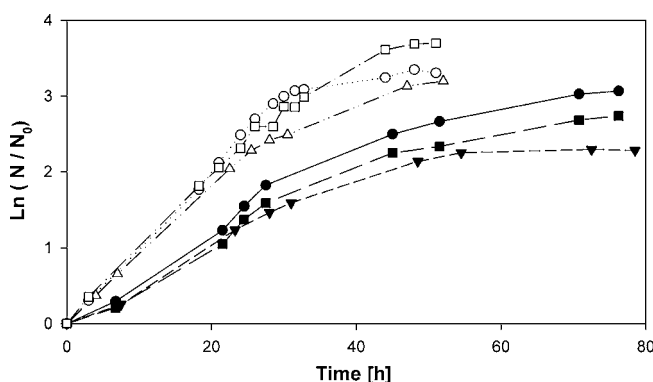
The growth behavior of *L. tarentolae* in the different nutrient media LEXSY Broth BHI, TSB and TB was determined in static suspension cultures, which are used

to provide sufficient cell counts for growth experiments and agitated cultures. Kinetics were determined after at least four sub-passages in the new medium under static conditions to allow adaptation of the cells and measurement of a representative growth curve.

Fig. 1 clearly shows that each nutrient medium supported the growth of *L. tarentolae* and the cells could be cultivated in the media with reduced content of animal substances (TSB and TB). Mean specific cell division rates were  $0.063\text{--}0.054\text{h}^{-1}$  in static suspension cultures (see Table 3). Over the total cultivation time, 3.9–4.4 generations were reached.

In agitated cultures,  $\nu$  was approximately  $1.5\times$  higher than in the static suspension culture due to a better supply of oxygen in agitated liquids. Doubling times were between 6.7–7.7 h (Table 3) and remarkably low in comparison to literature values (Table 1). The exponential growth finished at  $2.6\text{--}3.65 \times 10^8 \text{ cells ml}^{-1}$ . At the end of cultivation, a maximal cell density ( $N_{\text{max}}$ ) of  $9 \times 10^8 \text{ cells ml}^{-1}$  could be obtained (TB-medium). In contrast to literature data, these values are extremely high, because  $2 \times 10^8 \text{ cells ml}^{-1}$  was reported by Meehan *et al.* (2000) as the highest cell density (BHI-medium).

Glucose was used as the primary carbon source in all media examined.  $Y_{N/S}$  was calculated according to the equation 3 neglecting the maintenance metabolism. During the exponential growth phase in agitated cultures,  $Y_{N/S}$  varied between  $1.1 \times 10^8$  and  $1.4 \times 10^8 \text{ cells g}^{-1}$  glucose (Table 3). The lowest value was noticed using TSB-medium, where cells consumed more glucose for biomass production than in the other media. Probably



**Figure 1.** Growth kinetics of *L. tarentolae* in the nutrient media LEXSY Broth BHI (●), TSB (▼) and TB (■) in static suspension (filled symbols) and agitated culture (unfilled symbols) at  $26^\circ\text{C}$ . Static suspension cultures (10 ml) were cultivated in  $25 \text{ cm}^2$  cell culture flasks. Cell density  $N_0$  was  $2.46 \times 10^7 \text{ cells ml}^{-1}$  (LEXSY Broth BHI),  $2.96 \times 10^7 \text{ cells ml}^{-1}$  (TSB) and  $2.88 \times 10^7 \text{ cells ml}^{-1}$  (TB). Growth was determined in agitated cultures with 250 ml shaker flasks with baffles filled with 50 ml medium, 140 rpm. Cell density  $N_0$  was  $2.46 \times 10^7 \text{ cells ml}^{-1}$  (LEXSY Broth BHI),  $2.67 \times 10^7 \text{ cells ml}^{-1}$  (TSB) and  $2.28 \times 10^7 \text{ cells ml}^{-1}$  (TB).

**Table 3.** Growth parameters of *L. tarentolae* during exponential growth in various nutrient media, static suspension and agitated culture, kinetics see Fig. 1.

| Culture type | Medium          | $\nu$<br>[h <sup>-1</sup> ] | $t_D$<br>[h] | $k$<br>[-] | $Y_{NIS}$<br>[cells g <sup>-1</sup> glucose] | pH-shift<br>down |
|--------------|-----------------|-----------------------------|--------------|------------|--|------------------|
| static       | LEXSY Broth BHI | 0.063                       | 11.0         | 2.6        | $8.9 \times 10^7$                            | 7.6 → 7.0        |
|              | TSB             | 0.054                       | 12.8         | 2.4        | $9.1 \times 10^7$                            | 7.1 → 6.0        |
|              | TB              | 0.056                       | 12.4         | 2.4        | $1.2 \times 10^8$                            | 7.3 → 7.2        |
| agitated     | LEXSY Broth BHI | 0.103                       | 6.7          | 3.9        | $1.4 \times 10^8$                            | 7.6 → 6.9        |
|              | TSB             | 0.090                       | 7.7          | 3.3        | $1.1 \times 10^8$                            | 7.1 → 6.1        |
|              | TB              | 0.097                       | 7.1          | 3.6        | $1.3 \times 10^8$                            | 7.3 → 7.0        |

maintenance metabolism affected the calculation to a greater extent in this medium.

Consumption of glucose was accompanied by a decrease in pH resulting in changed physiological conditions in the media. The extent of the pH-shift differed between the media, seen in Table 3, and was largest in the TSB-medium, where a minimal level of pH 6.33 was achieved. Stabilization of the pH in the neutral range resulted in development of the TB-medium with a phosphate buffer system, where the pH drop could be limited to 6.9.

The physiological and morphological appearance of the cells in different media during exponential growth was similar.

When glucose was exhausted in all media, the cells switched over to alternative energy sources probably amino acids. As a result, pH increased and  $\nu$  was strongly reduced in comparison to glucose consumption, because the cells were mainly in maintenance metabolism. The final pH was maximal in the range of 7.4 to 7.7 where the cells appeared to be very thin and partially degenerated. Furthermore, growth could not be reactivated by passing the cells into fresh medium.

Summarizing the previous results, *L. tarentolae* can be grown in various complex media with high and stable specific cell division rates. The weight content of animal-derived substances is reduced by 25% ( $^{W/W}$ ) in TSB and by 50% ( $^{W/W}$ ) in TB-medium in comparison to LEXSY Broth BHI. TB- and TSB-medium contain beside hemin only a tryptic digest of casein, the main protein of milk, as substances of animal origin. On the one hand, in general opinion milk is regarded as free of prion proteins and therefore safe for humans. On the other hand, prion proteins can be detected in milk by an enzyme immunoassay (Boesen 2005). Furthermore, problems with batch-to-batch variations of casein, reported by Mueller and Miller (1954), resulted in changing yields of tetanus toxin production. For those reasons, the casein content in the nutrient medium has to be eliminated.

### Long-term stability of the static suspension culture

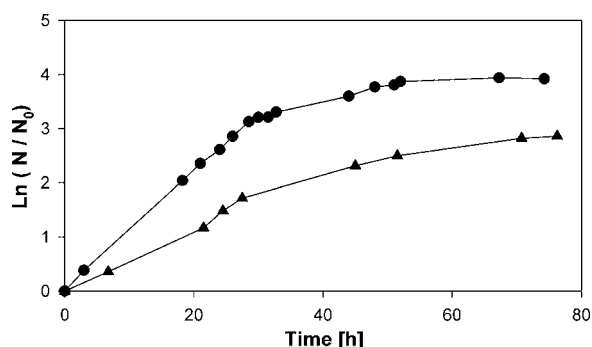
The growth performance of the static suspension culture in TB-medium was monitored over 70 sub-passages to ensure the stability of the cell material used for experiments with agitated cultures. The mean specific growth rate (determined over 2 days) was 0.043 h<sup>-1</sup> until passage 60. In passage number 70, cells failed to grow. Generally, static suspension culture can be used until passage 50 to ensure stable growth performance including a safety factor. Calculation of generation times resulted in more than 150 generations within 50 passages.

### Reduction of the TB-medium with “design of experiments”

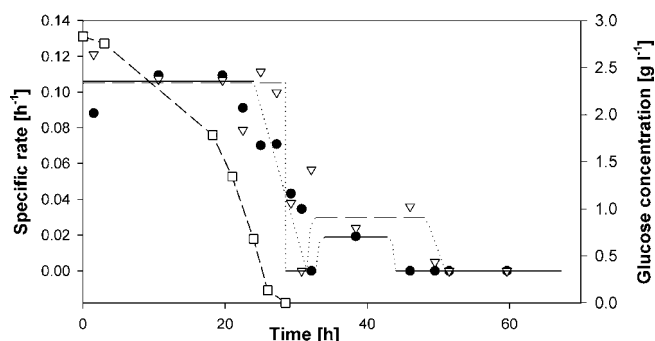
Detailed examinations of an effect to  $\mu$  by the single substances Bacto™ Yeast Extract and Bacto™ Tryptone of the TB-medium were conducted using the methods of “design of experiments” in a central composition experimental design (3<sup>2</sup>-design) in agitated cultures. Evaluation was done with a quadratic model and the method of the smallest squares of errors. The resulting equation (data not shown) significantly showed that Bacto™ Yeast Extract is the main factor in the TB-medium (6 times higher influence) and responsible for high specific growth rates. Influence of Bacto™ Tryptone is negligible, because it can be removed from the medium without drastic reduction of growth parameters. The newly developed YE-medium with consisting of Bacto™ Yeast Extract and buffer salts, has to be evaluated for stable growth.

### Alternative nutrient medium for cultivation of *L. tarentolae*

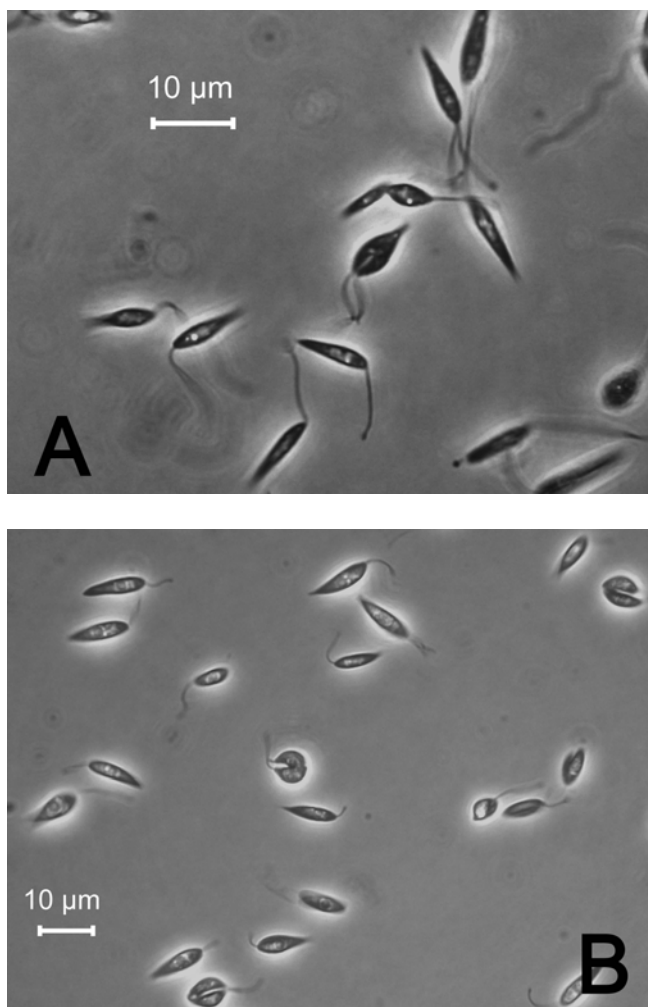
The YE-medium provides the opportunity to cultivate *L. tarentolae* in a medium containing only hemin as the substance of animal origin. In static suspension culture,  $\nu$  was 0.06 h<sup>-1</sup> (see Figure 2) and a stable growth over more than 50 passages could be found. A mean specific growth rate of 0.103 h<sup>-1</sup> ± 0.007 h<sup>-1</sup> was observed



**Figure 2.** Growth kinetics of *L. tarentolae* in YE-medium; (●) 250 ml shaker flask with baffles filled with 50 ml medium, cell density  $N_0 = 1.97 \times 10^7$  cells  $\text{ml}^{-1}$ ; (▲) static suspension culture with 10 ml medium in 25  $\text{cm}^2$  cell culture flasks,  $N_0 = 2.42 \times 10^7$  cells  $\text{ml}^{-1}$ .



**Figure 4.** The specific rates  $\mu$  (●) and  $\nu$  (∇) of *L. tarentolae* in YE-medium (250 ml shaker flask with baffles filled with 50 ml medium) between two measurement points are plotted to the average of the time intervals. Values of the logarithmic evaluation for  $\mu$  (—) and  $\nu$  (---) with assumed transition intervals (....) are shown. Furthermore, glucose kinetic is plotted (□).



**Figure 3.** Microscopic picture of *L. tarentolae* in A) LEXSY Broth BHI and b) YE-medium during exponential growth, phase contrast 2. Pictures were modified with levels adjustment to improve contrast and brightness across the entire picture.

in agitated cultures. Glucose was used as main carbon source and  $Y_{NIS}$  was calculated to  $1.18 \times 10^8$  cells  $\text{g}^{-1}$  glucose. Cells showed normal promastigote shape (seen in Fig. 3B) in comparison to cells in BHI-medium.

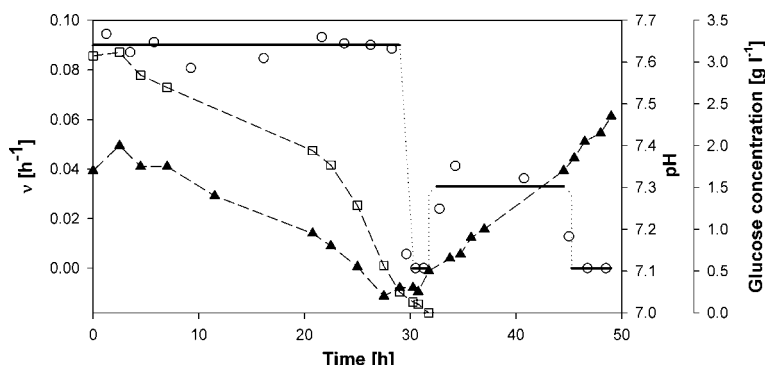
A correlation between cell density ( $N$ ) and optical density ( $OD$ ) at 600 nm wavelength was evident during exponential growth and in the early stationary phase. Therefore the correlation  $N \sim OD \rightarrow x \sim N$  is allowed and  $\nu = \mu$  as Figure 1 demonstrates. Later on, the correspondence between these parameters failed due to morphological changes of the cells and  $\nu \neq \mu$ .

During consumption of glucose, the decline in pH was limited to pH 7.0 as an effect of the salt buffer system used. More than 4.1 generations were observed during exponential growth with a maximal cell density of  $3.4 \times 10^8$  cells  $\text{ml}^{-1}$ , increasing to  $1 \times 10^9$  cells  $\text{ml}^{-1}$  and 5.7 generations when cultivation was finished.

When glucose was exhausted in the medium, pH increased continuously and reached a maximal value of 7.85, when cultivation was terminated. After observing the cells under the microscope, morphological changes attracted attention. The cells appeared to be very thin, needle-like, and partially degenerated in shape and size. Possible reasons for this could be that pH was moving out of physiological range or the extreme age of the culture.

### Bioreactor cultivation

The YE-medium was examined for its usability for bioreactor cultivations. The resulting plot is shown in Figure 5. During glucose consumption,  $\nu$  was  $0.092 \text{ h}^{-1}$  and  $Y_{NIS} = 1.6 \times 10^8$  cells  $\text{g}^{-1}$  glucose. Limitation of glucose was accompanied by an increase in pH, corresponding to the observations in static and agitated cul-



**Figure 5.** Batch fermentation of *L. tarentolae* in a 2 l bioreactor (YE-medium, 26 °C). Plot of  $v$  shows the value of the logarithmic evaluation (—) with assumed transition intervals (---) and the calculated values between measurement points plotted to the average of the time interval (○). Furthermore, the pH (▲) and the glucose (□) kinetics are shown.

ture. Maximal cell density of  $8.5 \times 10^8$  cells  $\text{ml}^{-1}$  was achieved.

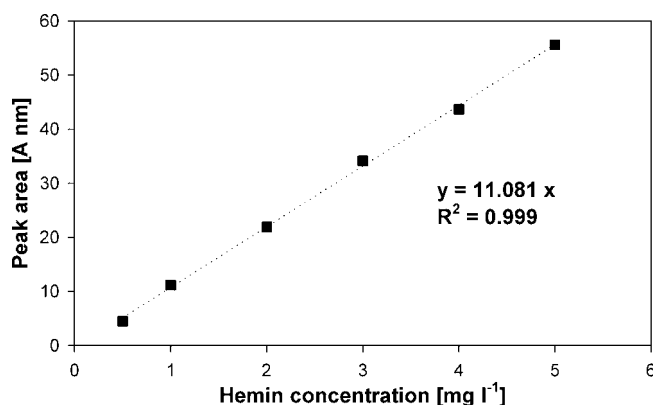
Summarizing the results, we have established a nutrient medium for *L. tarentolae* cultivation, which is serum-free and contains only hemin as substance of animal origin. The main component Bacto™ Yeast Extract is regarded as non-animal product by regulatory authorities (Bacto™ manuals, [www.bd.com/ds/technicalCenter/inserts/difcoBblManual.asp](http://www.bd.com/ds/technicalCenter/inserts/difcoBblManual.asp), Becton Dickinson, USA, 10.04.2006). Yeast Extract is a concentrate of the total water-soluble fraction of autolysed *Saccharomyces cerevisiae* cells. The content of vitamins (B-group), nitrogen, amino acids and carbon is very high. Commonly Yeast Extract is used in microbial cultivation of bacteria, yeast, cell and insect cultures. *L. tarentolae* can be grown stably and reproducibly with doubling times of 6.7 h in the YE-medium (Fritsche *et al.* 2006).

### EGFP-expression studies

Production of EGFP was analyzed at the end of exponential growth in agitated cultures by SDS-PAGE and determined as lane-purity (in %). The EGFP-expression was similar between the various media in the range of 12.5–16.8 lane% ( $\mu = 0.068\text{--}0.115 \text{ h}^{-1}$ ). The expression of EGFP is constitutive (chromosomal integration of one copy) and the growth-dependent with special correlation between  $\mu$  and the product formation. However, this had to be studied more detailed.

### Determination of hemin consumption

The determination of hemin was performed with an aqueous 2-phase-extraction using acidified chloroform (Lombardo *et al.* 2005). A good correlation between the hemin concentration and the peak area (between 340–450 nm, neutralized base line) is visible, as the standard curve in Figure 6 shows. In protein-rich solu-



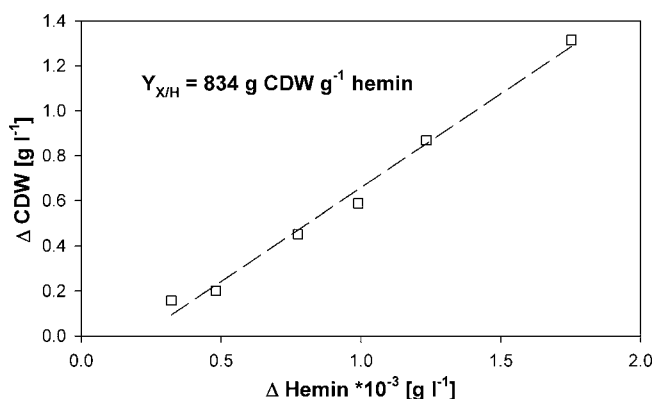
**Figure 6.** Standard curve for the hemin detection (—■—) with a correlation coefficient of 11.081  $\text{A nm l mg}^{-1}$  (peak area per hemin concentration) determined with a  $5 \text{ mg l}^{-1}$  hemin solution (in 0.5% triethanolamine).

tions, hemin adheres to the proteins and is coprecipitated by chloroform. Therefore, DMSO had to be added prior to extraction (Lombardo *et al.* 2005). The systematic influence of the proteins was analyzed in the YE-medium. The detection was limited to an offset of  $\sim 0.6 \text{ mg l}^{-1}$  of hemin (assuming constant protein content), because hemin could not be removed totally from the proteins. The correlation factor between hemin and the peak area could be kept constant.

$Y_{X/H}$  was calculated to  $834 \text{ g CDW g}^{-1}$  hemin for the YE-medium during exponential growth ( $\mu = 0,121 \text{ h}^{-1}$ ), seen in Fig. 7, and computed to  $Y_{N/H} = 1.1 \times 10^{14}$  cells  $\text{g}^{-1}$  hemin.

## Discussion

We have demonstrated that *L. tarentolae* can be cultivated in various nutrient media with comparable growth characteristics in static suspension and agitated



**Figure 7.** Yield coefficient  $Y_{X/H} = 834 \text{ g CDW g}^{-1} \text{ hemin}$  determined as correlation between cell dry weight and hemin concentration with the 2-phase-extraction during exponential growth in YE-medium.

cultures. The animal-derived components in the media have been reduced. First, the LEXSY Broth BHI was used, which consists of pulverized bovine brain and heart infusion. The media TSB and TB were intermediate steps, containing beside hemin only casein hydrolysate as ingredients of animal origin. Finally, the YE-medium was developed and evaluated, which is serum-free and contains only hemin as substance of animal origin. The static suspension culture was stable over more than 50 passages in the TB- and YE-medium. In LEXSY Broth BHI, no termination of growth was detectable, but this was not studied in detail.

In all media physiological conditions changed during growth, because consumption of glucose was accompanied by a decrease in pH. Van Hellemond *et al.* (1998) described, that Trypanosomatids excreted mainly partially oxidized products, like pyruvate, succinate and acetate during the energy metabolism. Especially abundance of nutrients, when cells consume more glucose than they can catabolize, leads to production of organic acids. Particularly the production of acetate is regarded as an overflow metabolism, comparable with the production of lactate or ethanol in other organisms.

The extent of the pH-shift differed between the media. The lowest value was detected in the TSB-medium (pH 6.0). *Leishmania* species are highly adaptable to different external pH values because they are exposed to extreme environmental changes (pH and temperature) during their life cycle. Differentiation of promastigotes to amastigotes was induced by a pH shift to an acidic environment (4.5–5.0) in combination with an increase in temperature (Zilberstein and Shapira 1994). Zilberstein *et al.* (1989) and Glaser *et al.* (1988) reported the ability of *L. donovani* to maintain the intracellular pH close to neutral over a wide range of environmental pH (5.0–7.4). The conclusion therefore is that the observed

pH shifts in LEXSY Broth BHI and TSB-medium are in the physiological range. On the other hand, an optimal pH for membrane-associated transport and metabolism of promastigotes was described in the range of pH 7.0–7.5 (Zilberstein and Shapira 1994). For this reason, we tried to stabilize the pH in the neutral range and developed the TB- and YE-medium with a phosphate buffer system, where the pH drop could be limited to pH 6.9. The better growth performance compared with TSB-medium confirmed this step.

After glucose consumption, pH raised and resulted finally in morphological changes of the cells, which appeared to be very thin and partially degraded in YE-medium. In contrast, Glaser *et al.* (1988) observed swollen cells, when the external pH was greater than 7.5, because promastigotes lost the ability to maintain cytosolic pH in the physiological range. Further investigations are needed to define the optimal pH conditions for cultivation of *L. tarentolae* in a biotechnological production process.

Resulting cell densities in all examined media were extremely high in comparison to literature data (Table 1). During exponential growth cell densities  $>2.6 \times 10^8 \text{ cells ml}^{-1}$  and a total amount of  $6.5 \times 10^8 - 1 \times 10^9 \text{ cells ml}^{-1}$  could be easily obtained. Compared to the highest value from literature, which was reported for BHI-medium (Meehan *et al.* 2000), 3.5 times higher cell densities were achieved in LEXSY Broth BHI, in TB- and YE-medium even 4.5 to 5 higher cell densities could be attained.

The calculated growth parameters from the literature in Table 1, like  $N_{\max}$ ,  $\nu$ , doubling time ( $t_D$ ) and generations ( $k$ ) do not indicate, if the cell size is changing in dependence on the specific cell division rate  $\nu$ . The counted parameters  $\nu$ ,  $t_D$  and  $k$  were determined under the assumption, that the cells are morphological equal in dependency on  $\nu$  and time. Our studies allow the conclusion that the cell size is significantly reduced if  $\nu \neq \mu$ . occurs. This is clarified in Figure 4 at time  $\geq 35 \text{ h}$ .

The observed doubling times (6.7 h, YE-medium) are exceptionally small in comparison to mammalian cell cultures, where the cells double approximately once a day. These facts improve the potential of the *L. tarentolae* expression system as an alternative to commonly used cell cultures.

With the expression of EGFP, the usability of the YE-medium for expression of a recombinant protein could be proved. Furthermore, the adaptation of the cultivation to a 2 l bioreactor was successful. During the bioreactor cultivation, no shear stress sensitivity was observed at the process parameter settings used. This



confirms our previous observations (Fritsche and Pohl 2006).

The consumption of the essential supplement hemin could be monitored by a 2-phase-extraction method. The estimated yield coefficient is extremely high in comparison to the consumption of glucose, showing a low demand of this supplement. Hemin is an essential component for growing *Leishmania* species, but the detailed function inside the cell is currently unknown. It is important as prosthetic group of various proteins, a source of energy and essential as an intracellular regulator for metabolic pathways involved in respiration and protein synthesis (Pal and Joshi-Purandar 2001, Srivastava *et al.* 1997). With this analytical tool, an essential component for growth could be monitored and this facilitates new possibilities for further bioprocess strategies.

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