A Fundamental Research of Growth, Metabolism and Product Formation of Tobacco Suspension Cells at Different Scales

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Abstract

For over two decades, plant cell cultures have been promising hosts for the expression of recombinant proteins such as hormones, growth factors, full-size antibodies and antigens. So far, over 700 different plant cell cultures are stored in the German Collection of Microorganisms and Cell Cultures (DSMZ) in Braunschweig. Among these plant cell cultures, the tobacco cell line *Nicotiana tabacum* L. cv. Bright Yellow 2 (BY-2) was chosen as a good host cell line for the production of recombinant proteins, as this cell line is well characterized – showing high growth rates and high cell synchrony.

Up to now, individual studies have only handled one or two parameters (i.e. biomass, osmolality or conductivity) for studying BY-2 cell growth. Such limited studies, however, do not provide a comprehensive insight into BY-2 cell metabolism. A first objective of this thesis, is to identify the optimal growth conditions for tobacco suspension cultures in shake flasks and to comprehensively characterize the growth of a transgenic BY-2 cell line. Hereby, multiple growth parameters were analyzed offline and online by using a Respiration Activity MOnitoring System (RAMOS). A faster shaking frequency resulted in clearly higher oxygen transfer rates and biomass concentrations. Moreover, a reproducibly observed shift of the oxygen transfer rate (OTR) could be identified to indicate ammonium depletion in the medium.

Today, the MS-medium is the preferred medium for the cultivation of tobacco suspension cells, even though it was formulated for an optimal growth and not for the production of recombinant proteins. Here, the fluorescent proteins GFP and YFP are used as model proteins and their expression was elucidated in detail. Based on the correlations between nutrient consumption, cell growth and product formation, it is the intention to improve the standard MS-medium to enhance the expression of the recombinant proteins. The initial ammonium concentration was found to have significant influence on either cell growth and played a pregnant role in protein synthesis. After the MS-medium was improved, the GFP concentration nearly doubled. When this improved ammonium enriched medium was applied to another transgenic tobacco cell line similar improvements to the amount of the glycoprotein influenza hemagglutinin (HA) produced by *Nicotiana tabacum* NT-1 cells could be achieved. Furthermore, a controlled-release system was successfully applied to plant suspension cultures. Using this controlled-release system where additional ammonium was supplied to the plant cells, an increase of 40% GFP intensity was observed.
Plant cells are maintained in suspension by pipetting a certain volume of grown culture into fresh medium. Applying this subcultivation method, results in non-defined growth conditions of plant cells. Due to that problem, plant suspension cultures always have to compete with animal cultures for the production of therapeutically relevant proteins as they have the advantage of an established cell banking system. Moreover, researchers are facing the huge problem of genetic instability of plant cells where growth and recombinant protein production tremendously vary. This well-known problem has been poorly documented so far. This growth variability of plant suspension cells was identified by relating the measured values of 22 oxygen transfer rates in a period of over two years. After the implementation of a new subculturing method a significantly better reproducibility of plant cell growth was obtained. However, the productivity, detected by fluorescence and Western blot, decreased by 80% in the same period.

Besides the cultivation of plant cells in shake flasks, in this work, plant suspension cells were also cultivated in stirred tank reactors and microtiter plates (MTPs). As there are no geometric similarities between shake flasks and stirred tank reactors, a scale-up is not a trivial process. Here, the scale-up was carried out under the assumption of a constant volumetric power input. It could be shown, that an increasing viscosity was the key parameter influencing cell growth in the fermenter. According to literature there is only one publication dealing with plant cell cultivation in small scale. One main drawback of cultivation in small scale is often insufficient oxygen supply and insufficient mixing. Here, BY-2 cell growth as well as GFP expression was monitored online using the BioLector technology. In addition, it was demonstrated that medium modifications influenced plant cell growth and the GFP production in the same way as shown in shake flasks.

Ultimately, this thesis provides a deep insight into tobacco cultivations in shake flasks, fermenters and MTPs. The combined offline and online analysis of tobacco suspension cultures was used for a detailed growth characterization and media optimization to improve growth and boost target product formation. In conclusion, the RAMOS technology allows the online analysis of oxygen consumption and has been proven to be a useful analytical tool investigating plant suspension cultures.
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Non potest fieri, ut non aliquando succedat multa temptanti.

Notwendigerweise hat einmal Erfolg, wer vieles versucht.

(Seneca, Epistulae morales 29,2)
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<th>Description</th>
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<td>(A_R)</td>
<td>reactor cross section area</td>
<td>([\text{m}^2])</td>
</tr>
<tr>
<td>(CTR)</td>
<td>carbon dioxide transfer rate</td>
<td>([\text{mol/L/h}])</td>
</tr>
<tr>
<td>(d_S)</td>
<td>inner shake flask diameter</td>
<td>([\text{m}])</td>
</tr>
<tr>
<td>(d_R)</td>
<td>blade diameter</td>
<td>([\text{m}])</td>
</tr>
<tr>
<td>(d_0)</td>
<td>shaking diameter</td>
<td>([\text{m}])</td>
</tr>
<tr>
<td>(D_R)</td>
<td>fermenter diameter</td>
<td>([\text{m}])</td>
</tr>
<tr>
<td>(g)</td>
<td>gravitational acceleration</td>
<td>([\text{m/s}^2])</td>
</tr>
<tr>
<td>(h)</td>
<td>height of an agitator blade</td>
<td>([\text{m}])</td>
</tr>
<tr>
<td>(H_R)</td>
<td>fermenter height</td>
<td>([\text{m}])</td>
</tr>
<tr>
<td>(i)</td>
<td>the van’t Hoff factor (number of individual particles of a compound dissolved in solution)</td>
<td>[-]</td>
</tr>
<tr>
<td>(k)</td>
<td>Metzner-Otto constant</td>
<td>[-]</td>
</tr>
<tr>
<td>(K)</td>
<td>flow consistency index</td>
<td>([\text{Pa·s}^m])</td>
</tr>
<tr>
<td>(L)</td>
<td>empirical coefficient</td>
<td>[-]</td>
</tr>
<tr>
<td>(L_1)</td>
<td>bottom clearance of 1st stirrer</td>
<td>([\text{m}])</td>
</tr>
<tr>
<td>(m)</td>
<td>flow behavior index</td>
<td>[-]</td>
</tr>
<tr>
<td>(M)</td>
<td>molarity of the solution</td>
<td>([\text{mol/L}])</td>
</tr>
<tr>
<td>(n_a)</td>
<td>agitation rate at which gas recirculation above the stirrer occurs</td>
<td>([1/\text{s}])</td>
</tr>
<tr>
<td>(n_b)</td>
<td>agitation rate at which gas recirculation below the stirrer occurs</td>
<td>([1/\text{s}])</td>
</tr>
<tr>
<td>(n_{bl})</td>
<td>number of blades</td>
<td>[-]</td>
</tr>
<tr>
<td>(n)</td>
<td>moles of oxygen</td>
<td>([\text{mol}])</td>
</tr>
<tr>
<td>(n_{st})</td>
<td>number of stirrer blades</td>
<td>[-]</td>
</tr>
<tr>
<td>(n_S)</td>
<td>shaking frequency</td>
<td>([1/\text{s}])</td>
</tr>
<tr>
<td>(n_R)</td>
<td>agitation rate</td>
<td>([1/\text{s}])</td>
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<tr>
<td>(OTR)</td>
<td>oxygen transfer rate</td>
<td>([\text{mol/L/h}])</td>
</tr>
<tr>
<td>(\Delta pO_2)</td>
<td>difference of oxygen partial pressure</td>
<td>([\text{bar}])</td>
</tr>
<tr>
<td>(P)</td>
<td>power</td>
<td>([\text{W}])</td>
</tr>
<tr>
<td>(P_g)</td>
<td>power input</td>
<td>([\text{W}])</td>
</tr>
<tr>
<td>(P_0)</td>
<td>unaerated power input</td>
<td>([\text{W}])</td>
</tr>
<tr>
<td>(P/V_L)</td>
<td>volumetric power input</td>
<td>([\text{W/m}^3])</td>
</tr>
</tbody>
</table>
Specific terms and units:

- Specific aeration rate ($q_{in}$) [vvm]
- Volumetric gas flow rate ($Q_G$) [m$^3$/min]
- Universal gas constant ($R$) [bar · L/mol · K]
- Temperature ($T$) [K]
- Time of measuring phase ($t$) [h]
- Superficial gas velocity ($u_g$) [m/s]
- Gas volume ($V_G$) [L]
- Liquid filling volume ($V_L$) [L]
- Molar volume of ideal gas ($V_{mol}$) [L/mol]
- (22.414 L/mol) at 0°C, 1.013 bar
- Fermenter volume ($V_R$) [L]
- Oxygen mole fraction of the supply gas ($y_{O2,in}$) [mol/mol]
  (with air: 0.2095%)
- Oxygen mole fraction of exhaust gas ($y_{O2,out}$) [mol/mol]
- Carbon dioxide mole fraction of inlet gas ($y_{CO2,in}$) [mol/mol]
  (with air: 0.035%)
- Carbon dioxide mole fraction of exhaust gas ($y_{CO2,out}$) [mol/mol]

Dimensionless Numbers:

- Axial Froude number ($Fr_a$) [-]
- Power number for fermenters ($P_o$) [-]
- Modified Power number for shake flasks ($P_o'$) [-]
- Phase number ($Ph$) [-]
- Reynolds number ($Re$) [-]
- Liquid film Reynolds number ($Re_f$) [-]

Greek Symbols:

- Effective shear rate ($\gamma_{eff}$) [1/s]
- Representative shear rate ($\gamma_{rep}$) [1/s]
- Dynamic viscosity ($\eta$) [Pa·s]
- Effective viscosity ($\eta_{eff}$) [Pa·s]
- Wavelength ($\lambda$) [nm]
- Osmotic pressure ($\pi$) [bar]
- Density ($\rho$) [kg/m$^3$]
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BY-2</td>
<td><em>Nicotiana tabacum</em> L. cv. Bright Yellow 2</td>
</tr>
<tr>
<td>CaMV</td>
<td>Cauliflower mosaic virus</td>
</tr>
<tr>
<td>CsVMV</td>
<td>Cassava vein mosaic virus</td>
</tr>
<tr>
<td>CTR</td>
<td>Carbon dioxide transfer rate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmatic reticulum</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GMP</td>
<td>Good manufacturing practice</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin A</td>
</tr>
<tr>
<td>HN</td>
<td>Hemagglutinin-neuraminidase</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>IFE</td>
<td>Inner filter effect</td>
</tr>
<tr>
<td>MTP</td>
<td>Microtiter plate</td>
</tr>
<tr>
<td>NT-1</td>
<td><em>Nicotiana tabacum</em> 1</td>
</tr>
<tr>
<td>OTR</td>
<td>Oxygen transfer rate</td>
</tr>
<tr>
<td>PCV</td>
<td>Packed cell volume</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PMPs</td>
<td>Plant-made pharmaceuticals</td>
</tr>
<tr>
<td>RAMOS</td>
<td>Respiration activity monitoring system</td>
</tr>
<tr>
<td>RQ</td>
<td>Respiratory quotient</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamid gel electrophoresis</td>
</tr>
<tr>
<td>USDA</td>
<td>U.S. Department of Agriculture</td>
</tr>
<tr>
<td>2,4-D</td>
<td>2,4-dichlorophenoxyacetic acid</td>
</tr>
</tbody>
</table>
Chapter 1  Introduction

1.1 Plant cells as a production system for recombinant proteins

Plant suspension cell cultures provide an alternative production platform for animal and human therapeutics, since the first recombinant protein was expressed in a plant cell culture in the early nineties (SIMONS ET AL. 1990). After this breakthrough the number of recombinant proteins expressed in plants has steadily increased and different transgenic plant cell suspension cultures have been used for producing recombinant proteins; these plant suspension cultures include rice (HUANG AND MCDONALD 2009), soybeans (SMITH ET AL. 2002), tomatoes (KWON ET AL. 2003) and tobacco (FISCHER ET AL. 1999A). The tobacco cell line Nicotiana tabacum L. cv. Bright Yellow 2 (BY-2), first isolated by KATO ET AL. (1972), was chosen as a good host cell line for plant suspension cultures, since this cell line is well characterized – showing high growth rates and high cell synchrony (NAGATA ET AL. 1992). According to COMBETTES ET AL. (1999) and DAVID AND PERROT-RECHENMANN (2001), this cell line is the model cell line for studying of plant cell cycles and investigating the cellular biology of plants.

Up to now, a number of various recombinant proteins, such as antibody fragments (FIREK ET AL. 1993; FISCHER ET AL. 1999A), full-size antibodies (FISCHER ET AL. 1999B; SACK ET AL. 2007), enzymes (SCHIERMEYER ET AL. 2005; SCHINKEL ET AL. 2005; SORRENTINO ET AL. 2005) and even mature immunologically active allergens (LIENARD ET AL. 2007) have been produced in BY-2 cells. The production of recombinant proteins from transgenic suspension cultures offers many advantages over that from intact transgenic plants including a better process control and fast generation of transgenic cell lines (FISCHER ET AL. 1999C). A further advantage is that the target protein can be potentially secreted to the culture broth which facilitates the separation and purification of the respective protein. In addition, plant cell cultures are not exposed to agrochemicals and variable cultivation conditions due to changes of weather and other environmental conditions (HELLWIG ET AL. 2004) and, moreover, plant cells have a well-documented ability to perform post-translational modifications (GOMORD AND FAYE 2004). However, plant suspension cells are generally characterized by low protein
expression levels of 1 – 10 mg/L, although a production level of 129 mg/L was reported for transgenic rice cell suspensions (SHIN ET AL. 2003).

In 2006, the company Dow AgroSciences (Indianapolis, USA) has received the regulatory approval for the world’s first plant-cell-produced vaccine from the U.S. Department of Agriculture (USDA) (TRAVIS 2008). The approved recombinant protein was a veterinary vaccine based on the hemagglutinin-neuraminidase (HN) antigen of the Newcastle disease virus. A further successful example for the production of a therapeutically relevant protein by Dow AgroSciences includes the hemagglutinin A (HA) antigen of avian influenza (MIHALIAK ET AL. 2007). A comprehensive overview of plant cell-produced recombinant proteins was recently reviewed by HUANG AND MCDONALD (2009). To date, several companies are focusing on the production of plant-made pharmaceuticals (PMPs) as shown in Table 1-1.

Table 1-1: Current status of plant-made pharmaceuticals (PMPs) in clinical trials produced by various companies. The table was first published by (FOX 2006) and last updated July 2011 from http://www.molecularfarming.com/PMPs-and-PMIPs.html

<table>
<thead>
<tr>
<th>Company</th>
<th>Source</th>
<th>Protein</th>
<th>Trial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protalix Therapeutics</td>
<td>Carrot</td>
<td>Glucocerebrosidase against Gaucher’s disease</td>
<td>On Sale - named patient basis</td>
</tr>
<tr>
<td>Bayer Innovation</td>
<td>Tobacco</td>
<td>Anti-idiotypic IgG antibodies against Non-Hodgins lymphoma</td>
<td>Phase I</td>
</tr>
<tr>
<td>Biolex Therapeutics</td>
<td>Duckweed</td>
<td>Alpha-interferon against hepatitis C</td>
<td>Phase IIb, formulated dose</td>
</tr>
<tr>
<td>Chlorogen</td>
<td>Tobacco</td>
<td>TGF-beta</td>
<td>Advanced animal trials</td>
</tr>
<tr>
<td>Cobento</td>
<td>Arabidopsis</td>
<td>B-12 deficiency</td>
<td>Successful 37 patient clinical trial, cGMP production certified</td>
</tr>
<tr>
<td>Guardian Biotechnologies</td>
<td>Canola</td>
<td>Vaccine against coccidiosis</td>
<td>Phase II</td>
</tr>
<tr>
<td>Medicago</td>
<td>Tobacco</td>
<td>Vaccine against pandemic and seasonal influenza</td>
<td>Phase II</td>
</tr>
<tr>
<td>Meristem Therapeutics</td>
<td>Corn</td>
<td>Lactoferrin against gastrointestinal disorder</td>
<td>Phase I</td>
</tr>
<tr>
<td>Meristem Therapeutics</td>
<td>Corn</td>
<td>Lipase against cystic fibrosis</td>
<td>Phase II</td>
</tr>
<tr>
<td>Nexgen Biotechnologies</td>
<td>Melon</td>
<td>Hormone receptor for diagnosis of Graves disease</td>
<td>Available from company</td>
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<tr>
<td>Planet Biotechnology</td>
<td>Tobacco</td>
<td>CaroRx for tooth decay</td>
<td>Phase II, already granted an EU license as a medical device</td>
</tr>
<tr>
<td>SemBioSys</td>
<td>Safflower</td>
<td>Insulin</td>
<td>Phase III</td>
</tr>
<tr>
<td>Ventria Bioscience</td>
<td>Rice</td>
<td>Infant formula enhancer</td>
<td>On sale to infant formula makers</td>
</tr>
</tbody>
</table>
Further information regarding the phase of the clinical trials was recently published by Paul and Ma (2011). According to Table 1-1, industrial processes involving plant cell cultures have been limited to only a handful of applications. In 1993, the company Phyton Biotech installed the worlds-largest cGMP plant cell culture facility in Ahrensburg, Germany to produce paclitaxel, an anticancer secondary metabolite, in a 75,000 L scale.

1.2 Plant cell metabolism

In vitro cultures of heterotrophic plant cell suspensions require a variety of different nutrients and suitable physical conditions for their growth in shake flasks. The composition of an optimal plant tissue culture medium depends on the type of the plant cell culture. As no single medium can be used for all types of plant cells, the composition of the culture medium for each plant material has to be developed individually. These so-called “mineral” media generally consist of a defined concentration of various inorganic salts, amino acids, hormones and vitamins and have been empirically developed solely by trial and error. Subsequently, dose-response experiments have been conducted to adjust the optimal nutrient composition. For heterotrophic plant cell suspensions also a carbon source has to be added to the culture medium.

In order to clarify the variety of the plant cell mixtures, the company PhytoTechnology Laboratories (Kansas City, KS, USA) offers 121 different basal salt mixtures for plant cell cultivations. The most commonly used one is the MS-medium of Musharige and Skoog developed for the optimal growth of tobacco cells (Murashige and Skoog 1962). Other relevant mixtures are the Gamborg B5 medium for soybean cultures (Gamborg et al. 1968) and the N6-medium for cereal cultures (Chu et al. 1975).

1.2.1 Nitrogen metabolism

Nitrogen – which is the macronutrient with the highest concentration in the majority of all mineral media – is a constituent of both nucleic acids and proteins and is thus essential to plant life. Nitrogen is directly connected to amino acid and protein biosynthesis (Crawford 1995) and plays therefore a pivotal role in plant cell metabolism. Most commonly used nitrogen sources in media for cultivating plant cells are nitrate, ammonium, urea and amino acids. The MS-medium is characterized by a high amount of nitrogen and consists of a mixture of two different nitrogen sources in a defined ratio, namely nitrate and ammonium. Nitrate ions, are essential for plant cell growth and nearly all published media, including the MS-medium, provide the majority of their available nitrogen in this form. Interestingly,
nitrate cannot be directly utilized by the cells, as it has to be reduced to ammonium. The assimilation of nitrate and ammonium ions in plant cells is shown in Figure 1-1.

According to Figure 1-1, ammonium (NH$_4^+$) is a readily metabolizable source of nitrogen (marked with the dotted arrow) and is directly transformed into glutamine and glutamic acid by glutamine synthetase (GS) and glutamate synthase (GOGAT) (Padgett and Leonard 1993). However, cells cannot grow in a medium with ammonium as the sole nitrogen source (Gamborg and Shy Luk 1970). Explanations for that are the latent toxicity induced by an excess of ammonium ions and the need to control the pH of the medium (Mott et al. 1985). Without pH-control, the pH of the media containing ammonium as the sole nitrogen source falls rapidly to a point where no cell growth is possible (~ pH 4) (Dougall 1981). However, if Krebs cycle acids such as citrate, malate, fumarate or succinate are added to the MS-medium plant cell growth can be observed (Gamborg 1970). Nitrate, in contrast, has to be reduced to ammonium before being utilized (Figure 1-1). The reduction of nitrate to ammonium is a two-step reaction catalyzed by the nitrate reductase (NR) and by the nitrite reductase (NiR). This reduction is performed by the cell at the expense of 4 mol of NAD(P)H per mol of nitrate (Kaiser and Huber 1994). On the one hand the presence of nitrate induces both enzymes (Beevers and Hageman 1969; Filner et al. 1969); on the other hand...
ammonium and amino acids can inhibit the activity of these enzymes (FILNER 1966; JOY 1969). Thus, the pathway is regulated by its substrates and products. Some plants can grow in a medium with nitrate as sole nitrogen source, but BY-2 cells grow faster when the medium contains a mixture of nitrate and ammonium (GAMBORG 1970). Urea, however, can be used as a sole nitrogen source, but the growth is slower compared to a mixture of ammonium and nitrate (KIRBY ET AL. 1987).

1.2.2 Osmotic pressure

Besides acting as nutrients for the plant cells, solutions of salts and carbohydrates influence plant cell growth through their osmotic pressure. The osmotic pressure significantly affects plant cell growth and the cell morphology (GUO ET AL. 2005). Therefore, it is itself a major influencing parameter in plant suspension culture and is also used as an indicator for the growth of plant suspension cells (MADHUSUDHAN ET AL. 1995). Moreover, it influences the production of recombinant proteins (TERASHIMA ET AL. 1999) and plays an important role in the carbohydrate-modulated gene expression (KOCH 1996). In general, the osmotic pressure can be calculated according to the following equation:

\[ \pi = i \cdot M \cdot R \cdot T \]  

Eq.: 1-1

with

- \( \pi \) osmotic pressure [bar]
- \( i \) the van ’t Hoff factor (number of individual particles of a compound dissolved in solution) [-]
- \( M \) molarity of the solution [mol/L]
- \( R \) universal gas constant [bar \cdot L/mol \cdot K]
- \( T \) temperature [K]

The osmotic pressure is typically expressed as either osmoles/kilogram (Osm/kg) of solvent – referred to as osmolality, or osmoles/liter (Osm/L) of solution – referred to as osmolarity. Often, the calculated osmolarity is considered roughly equivalent to osmolality (i.e., 1 L ~ 1 kg) as plant media are dilute aqueous solutions with a specific density of ~ 1 (ERSTAD 2003). Throughout this work the term osmolality (Osm/kg) was used. Osmotic pressure contributes to the movement of the water through the plant cells. Figure 1-2 describes the three different phenomena of turgor pressure in plant cells.
If plant cells are given into a hypertonic solution water is extracted from the protoplasm and the volume of the vacuole shrinks (Figure 1-2A). This process is called plasmolysis. In an isotonic surrounding the osmotic pressure inside and outside of the cell is equal and the cell volume remains constant (Figure 1-2B). The process of plasmolysis (Figure 1-2A) is reversed as soon the cells are transferred into a hypotonic solution. Here, water is flushing into the vacuole. This phenomenon is called deplasmolysis (Figure 1-2C).

To study the effect of osmotic stress on the metabolism of plant cells, so-called inert osmolytes are used to artificially increase the osmotic pressure in the medium. For this purpose sugar alcohols as sorbitol or mannitol are typically used. Mannitol is an inert osmolyte which is not consumed by the cells (THOMPSON ET AL. 1986). It can easily penetrate cell walls but not the plasmalemma. Thus, this osmolyte cannot penetrate into the cells and, therefore, it inhibits the water uptake of the cells (RAINS 1989). By doing so the fresh weight of the cells decreases whereas the dry weight remains constant (BINZEL ET AL. 1985).

### 1.3 Online monitoring of plant cell growth in shake flasks

The cultivation of various plant suspension cultures in shake flasks and the characterization of their growth is state of the art in industry and academia. Unfortunately, there is no uniform description of plant cell growth. It can be either documented by invasive growth parameters (PCV, wet and dry weight, osmolality) or by non-invasive parameters (dissimilation curves, cell volume after sedimentation) determined by time course experiments (MUSTAFA ET AL. 2011; SCHRIPSEMA ET AL. 1990). Thereby, it is difficult to correlate the above mentioned parameters with limitations, inhibitions or the physiological state of the plant cell. Here, the measurement of the oxygen consumption of the cell can give a deeper insight into their metabolism since all metabolic steps are connected to oxygen. By using the Respiration
Activity Monitoring System (RAMOS) developed by ANDERLEI AND BÜCHS (2001), it is possible to determine online the oxygen transfer rate (OTR), the carbon dioxide transfer rate (CTR) and the respiratory quotient (RQ) of aerobic growing cells in shake flasks. This device has already been used to characterize plant cell growth of *A. indica* (RAVAL ET AL. 2003) and *W. australis* (RECHMANN ET AL. 2007). In addition, the system was used for various organisms including bacteria, yeast, fungi and even mammalian cells although the breathing activity of animal cells is up to 100-times lower compared to bacteria. An overview of the microorganisms cultivated in RAMOS so far including references is summarized in Table 1-2.

### Table 1-2: Different organisms cultivated in the RAMOS device

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Corynebacterium glutamicum (SÉLETZKY ET AL. 2006; ZIMMERMANN ET AL. 2006)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Bacillus subtilis</em> (GÜEZ ET AL. 2008)</td>
</tr>
<tr>
<td></td>
<td><em>Azotobacter vinelandii</em> (PEÑA ET AL. 2011)</td>
</tr>
<tr>
<td></td>
<td><em>Escherichia coli</em> (ANDERLEI AND BÜCHS 2001; LOSEN ET AL. 2004)</td>
</tr>
<tr>
<td></td>
<td><em>Gluconobacter oxydans</em> (ANDERLEI AND BÜCHS 2001; SILBERBACH ET AL. 2003)</td>
</tr>
<tr>
<td></td>
<td><em>Streptomyces pristinaespiralis</em> (MEHMOOD ET AL. 2011)</td>
</tr>
<tr>
<td>Yeast</td>
<td><em>Arxula adenivorans</em> (STÖCKMANN ET AL. 2009)</td>
</tr>
<tr>
<td></td>
<td><em>Saccharomyces cerevisiae</em> (ANDERLEI AND BÜCHS 2001)</td>
</tr>
<tr>
<td></td>
<td><em>Hansenula polymorpha</em> (STÖCKMANN ET AL. 2003)</td>
</tr>
<tr>
<td></td>
<td><em>Schizosaccharomyces pombe</em> (KLEMENT ET AL. 2011)</td>
</tr>
<tr>
<td>Fungi</td>
<td><em>Botyis cinera</em> (ANDERLEI AND BÜCHS 2001)</td>
</tr>
<tr>
<td>Plants</td>
<td><em>Azadirachta indica</em> (RAVAL ET AL. 2003)</td>
</tr>
<tr>
<td></td>
<td><em>Wolffia australis</em> (RECHMANN ET AL. 2007)</td>
</tr>
<tr>
<td>Mammalian</td>
<td>Chinese hamster ovary cells (BISELLI ET AL. 2010)</td>
</tr>
<tr>
<td></td>
<td>Hybridoma cells (CANZONERI ET AL. 2006)</td>
</tr>
</tbody>
</table>

### 1.4 Scale-up of plant cells

As there are no geometric similarities between shake flasks and fermenters, for a scale-up, many aspects have to be considered. In general, it is impossible to scale-up a process from a shake flask to a fermenter while keeping all important process conditions constant. Thus, the critical criterion for the scale-up of a process has to be carefully chosen. Typical scale-up criteria are the volumetric power input (P/V_L), the volumetric mass transfer coefficient (k_L,a), and the impeller tip speed (MARGARITIS AND ZAJIC 1978).

In the last decades, various plant cell lines have been cultivated in totally different types of fermenters, i.e. bubble columns (KATO ET AL. 1974), wave bioreactors (EIBL AND EIBL 2008), and stirred tanks (BYRNE AND KOCH 1962). The most common used fermenter in industry is
the stirred tank. This fermenter is characterized by a high oxygen mass transfer ability, easy scale-up, and compliance with cGMP requirements. Moreover, alternative impellers can be used in this fermenter. Disadvantages are the high shear stress due to impellers, the higher risk of contamination due to a mechanical sealing of the bearing and the heat generation due to mechanical agitation (HUANG AND MCDONALD 2009). In this work, a stirred tank with a Rushton turbine was used.

Physical properties of a fermentation broth, such as cell morphology, density and viscosity have impact on the flow regime in the reactor and, thus, are necessary in the consideration of mixing and mass transfer. Plant cells are characterized by various properties which distinguish them from microbial cells. The most important differences of plant and microbial cells are summarized in Table 1-3 (TATICEK ET AL. 1991).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Microbial cell</th>
<th>Plant cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>≈ μm³</td>
<td>≥ 10⁵ μm³</td>
</tr>
<tr>
<td>Cell aggregation</td>
<td>often</td>
<td>normally in clumps</td>
</tr>
<tr>
<td>Doubling time</td>
<td>≤ 1 h</td>
<td>24 - 150 h</td>
</tr>
<tr>
<td>Inoculation density</td>
<td>small</td>
<td>5-20% of total volume</td>
</tr>
<tr>
<td>Shear stress tolerance</td>
<td>high</td>
<td>low</td>
</tr>
<tr>
<td>Water content</td>
<td>≈ 75%</td>
<td>≈ 95%</td>
</tr>
<tr>
<td>Oxygen consumption</td>
<td>10-110 mmol/L/h</td>
<td>1-10 mmol/L/h</td>
</tr>
<tr>
<td>Product</td>
<td>Intra- or extracellular</td>
<td>usually intracellular</td>
</tr>
<tr>
<td>Medium cost</td>
<td>≈ $6 per m³</td>
<td>≈ $50 per m³</td>
</tr>
</tbody>
</table>

Table 1-3: Comparison of the characteristics of microbial and plant cells

A very important factor to be considered in a scale-up of plant cells is the huge difference in the oxygen demand (Table 1-3). Oxygen is the most important gaseous substrate for the in vitro cultivation of plant cells and its consumption can be monitored by using the OTR. Due to their slow metabolism, the oxygen demand of plant cells is significantly lower than that of microbial cells. However, growth and productivity can be limited because of a bad oxygen mass transfer in the fermenter due to a high viscosity of the culture broth (LIU AND LEE 1999). Another important difference between microbial and plant cells is the fact that plant cells tend to form large cell cluster resulting from the secretion of extracellular polysaccharides. Such clumps can reach mm-scale depending on the cell line, fermenter type, inoculation density, cultivation conditions and the cultivation medium (KING 1977). In larger cell clumps, aggregation can lead to an inadequate transfer of nutrients and oxygen to the cells and, therefore, these cells can be oxygen/nutrient deficient. This inadequate cultivation conditions can result in an inferior cell growth and low recombinant protein production (TATICEK ET AL. 1991) or even hollow centers owing to autolysis of the inner cells (HULST ET AL. 1985).
Moreover, the progress of aggregation has been found to be the major contributor to the increasing viscosity during the cultivation. From a rheological point of view the culture broth exhibits a non-Newtonian behavior and is a pseudoplastic fluid (KATO ET AL. 1978). A similar behavior was found in cultivations of *Catharanthus roseus* cells (SCRAGG ET AL. 1986).

Especially for the scale-up of plant cells it is of prime importance to adjust fermenter configurations which can provide adequate mixing and mass transfer while minimizing the intensity of shear stress. Thus, problems arise when scaling up plant suspension cultures from shake flasks to large scale bioreactors.

### 1.5 Overview and objectives of this work

This thesis results from a close collaboration with the company Dow AgroSciences (Indianapolis, USA) and the Fraunhofer Institute for Molecular Biology and Applied Ecology (IME) (Aachen). This study aims to comprehensively characterize tobacco suspension cultures, in terms of growth, metabolism and recombinant protein production. Not only is the objective to characterize the tobacco cell growth in shake flasks, but also in a stirred tank fermenter and in microtiter plates (MTPs). Therefore, scale-up and scale-down experiments have been performed.

For an optimized production of recombinant proteins in shake flasks, at first, the optimal cultivation conditions have to be figured out. These experiments are performed with two different tobacco wild type cell lines while monitoring the oxygen transfer rate (OTR) and plant cell growth. Second, from 15 different transgenic cell lines the best producing cell line is identified on the basis of the fluorescence intensity (Chapter 3) and of important molecular biological aspects (i.e. regeneration of protoplasts). In Chapter 4, this work provides a comprehensive overview of cell growth and nutrient consumption of the chosen tobacco suspension cell line in shake flasks. To achieve this, multiple offline parameter (cell growth, nutrient consumption, protein production) are comprehensively analyzed over a 10-day cultivation. In addition to the offline parameters, the OTR of this cell line is monitored online.

Based on the knowledge obtained from the characterization in shake flasks, the next step – a medium modification – is realized by changing initial nitrogen concentrations in the commercial MS-medium (Chapter 5). To achieve this, the MS-medium is reproduced from stock solutions and the reproducibility compared to the commercial MS-medium is evaluated. Furthermore, this modification is subsequently transformed to another tobacco suspension culture. Also with this cell line the medium modification is tested. During the tobacco cell
growth analysis, special attention has been paid to the osmotic pressure of the culture broth (Chapter 6) as it has been identified as one of the key parameter in plant suspension culture.

After a fundamental analysis of tobacco cell growth in shake flasks, BY-2 cells are scaled-up to a 1.5 L laboratory-scale stirred tank fermenter (Chapter 7). The scale-up was performed on the basis of an identical initial power input. In addition, BY-2 cells are scaled-down to a 1000 µL volume in MTPs (Chapter 8). Thereby, different media modifications – applied already in shake flasks – are adopted to BY-2 cell growth in MTPs and are evaluated.

Based on the inoculation method, plant suspension cultures generally are subject to variations in cell growth. Thus, the growth reproducibility of a transgenic cell line is investigated (Chapter 9). To achieve this, the OTR determined in a period of 22 months is summarized and analyzed in terms of cell growth. Moreover, the stability of the recombinant protein production is evaluated.
Chapter 2  Material and Methods

2.1  Material

2.1.1  Chemicals, devices and consumables
The entire list of the chemicals, devices and consumables is attached in the Appendix.

2.1.2  Kits, antibodies and protein marker
Within this work, following kits have been used:

- Protein assay kit (Bio-Rad Laboratories Inc., Munich) to determine the total soluble protein concentration.

- Cuvette test kits for the determination of ammonium, nitrate, phosphate, potassium and sulfate (Merck KGaA, Darmstadt).

For the Western blot the following antibodies have been used:

- Rabbit-anti-TurboGFP (Evrogen JSC, Moscow, RUS): diluted 1:20,000 in PBS buffer.

- Goat-anti-Rabbit-IgG conjugated with alkaline phosphatase (Jackson ImmunoResearch Europe Ltd., Suffolk, UK): diluted 1:5,000 in PBS buffer.

- For the SDS-PAGE the protein marker PageRuler (Fermentas, St. Leon-Rot) was used.

2.2  Different plant cell lines
In this study, several different wild type and transgenic tobacco cell lines have been used:

Three different wild type cell lines of BY-2 and NT-1 cells:

- BY-2 wt (Fraunhofer Institute, IME, Aachen)
- BY-2 wt (Dow AgroSciences, Indianapolis, USA)
- NT-1 wt (Dow AgroSciences, Indianapolis, USA)

and four different transgenic cell lines of BY-2 and NT-1 cells:
Material and Methods

- BY-2\textsubscript{CsVMV}GFP-KDEL (Dow AgroSciences, Indianapolis, USA)
- BY-2\textsubscript{CmAct}YFP (Dow AgroSciences, Indianapolis, USA)
- NT-1\textsubscript{CsVMV}HA (Dow AgroSciences, Indianapolis, USA)
- BY-2\textsubscript{CaMV}M12 (Fraunhofer Institute, IME, Aachen)

The four transgenic cell lines used in this work were established completely at the Fraunhofer Institute (IME). Suspension cultures of the transgenic \textit{Nicotiana tabacum} BY-2 cell line expressing GFP were developed from wild type strain \textit{N. tabacum} BY-2. This wild type strain was transformed using \textit{Agrobacterium tumefaciens} (strain LBA4404) containing the vector pDAB100352. \textit{A. tumefaciens} harboring pDAB100352 was co-incubated with wild type \textit{N. tabacum} BY-2 suspension cultures. The transformed plant cells were selected using the herbicide imazethapyr. The T-DNA (transfer DNA) of pDAB100352 encodes the expression cassette for ER-retarded green fluorescent protein (GFP-KDEL) \textsc{Munro and Pelham 1987} under the control of the \textit{Cassava vein mosaic virus} promoter (CsVMV) \textsc{Verdaguer et al. 1998}. The YFP expressing BY-2 cell line was transformed in the same way harboring the vector pDAB9642. The T-DNA of pDAB9642 encodes the expression cassette for the yellow fluorescent protein (YFP) under the control of an actin promoter of \textit{Cucumis melo}. The transgenic \textit{N. tabacum} NT-1 cell line expressing the glycoprotein influenza hemagglutinin (HA) contains the plasmid described previously \textsc{Cardineau et al. 2009}. The expression of HA was also driven by the \textit{Cassava vein mosaic virus} promoter and the recombinant HA was targeted to the endomembrane system of the plant cell. The transgenic BY-2 cell line from the Fraunhofer IME expressing the antibody M12 was developed described by \textsc{Raven et al. (2010)}.

2.3 MS-medium

In the early sixties the MS-medium was developed by Murashige and Skoog for the growth of tobacco cultures \textsc{Murashige and Skoog 1962}. It is a mineral medium, consisting of defined amounts of various macro and micro nutrients and serves as a basal medium for the growth of tobacco cells. A detailed composition of the MS-medium is listed in the Appendix IV. For BY-2 and NT-1 cells, there were minor differences in the respective medium composition:

<table>
<thead>
<tr>
<th>MS-medium for BY-2 cells:</th>
<th>MS-medium for NT-1 cells:</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.43 g/L MSMO</td>
<td>4.43 g/L MSMO</td>
</tr>
<tr>
<td>30 g/L sucrose</td>
<td>30 g/L sucrose</td>
</tr>
<tr>
<td>0.2 g/L KH\textsubscript{2}PO\textsubscript{4}</td>
<td>0.137 g/L K\textsubscript{2}HPO\textsubscript{4}</td>
</tr>
<tr>
<td>0.6 mg/L Thiamine-HCl</td>
<td>1 mg/L Thiamine-HCl</td>
</tr>
<tr>
<td>0.2 mg/L 2,4-D</td>
<td>4.44 mg/L 2,4-D</td>
</tr>
<tr>
<td></td>
<td>0.5 g/L MES buffer</td>
</tr>
</tbody>
</table>
Before autoclaving (121°C, 21 min), the pH was adjusted to 5.8 with 1 M KOH in both media.

### 2.3.1 Self-made MS-medium by using stock solutions

In order to modify the MS-medium different stock solutions were prepared which enabled a variation of single compounds in the medium. The different stock solutions are shown in Table 2-1.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration in stock solution [mM]</th>
<th>Final concentration [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Halogen stock solution (100x)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCl&lt;sub&gt;2&lt;/sub&gt; · 2 H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>299.3</td>
<td>2.99</td>
</tr>
<tr>
<td>CoCl&lt;sub&gt;2&lt;/sub&gt; · 6 H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>0.01</td>
<td>1·10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>KI</td>
<td>0.5</td>
<td>5·10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Sulfate stock solution (100x)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgSO&lt;sub&gt;4&lt;/sub&gt; · 7 H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>150.1</td>
<td>1.5</td>
</tr>
<tr>
<td>MnSO&lt;sub&gt;4&lt;/sub&gt; · 4 H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>10</td>
<td>0.1</td>
</tr>
<tr>
<td>ZnSO&lt;sub&gt;4&lt;/sub&gt; · 7 H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>2.99</td>
<td>0.03</td>
</tr>
<tr>
<td>CuSO&lt;sub&gt;4&lt;/sub&gt; · 5 H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>0.01</td>
<td>1·10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>PbMo stock solution (100x)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KH&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>271.88</td>
<td>2.72</td>
</tr>
<tr>
<td>H&lt;sub&gt;3&lt;/sub&gt;BO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>10.03</td>
<td>0.1</td>
</tr>
<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;MoO&lt;sub&gt;4&lt;/sub&gt; · 2 H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>0.1</td>
<td>1·10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>NaFe-EDTA stock solution (200x)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;EDTA</td>
<td>10</td>
<td>0.1</td>
</tr>
<tr>
<td>FeSO&lt;sub&gt;4&lt;/sub&gt; · 7 H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>10</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Nitrate stock solution (100x)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH&lt;sub&gt;4&lt;/sub&gt;NO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>2061.47</td>
<td>20.61</td>
</tr>
<tr>
<td>KNO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>1879.33</td>
<td>18.79</td>
</tr>
<tr>
<td><strong>Vitamins / Hormones</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiamine-HCl</td>
<td>11.86</td>
<td>3·10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>2,4-D</td>
<td>18.1</td>
<td>9·10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td>myo-inositol</td>
<td>555.06</td>
<td>0.55</td>
</tr>
<tr>
<td><strong>Carbon source</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
<td>87.64</td>
</tr>
</tbody>
</table>

Listed stock solutions were diluted in deionized water and stored at 4°C. One exception was the 2,4-D stock solution. 2,4-D was diluted in dimethyl sulfoxide (DMSO) and stored at room temperature.

**For 1 L self-made MS-medium for BY-2 cells:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>mL of each stock solution (halogen, sulfate, PbMo, NaFe-EDTA and nitrate)</td>
<td>10</td>
</tr>
<tr>
<td>g/L sucrose</td>
<td>30</td>
</tr>
<tr>
<td>µL Thiamine-HCl</td>
<td>150</td>
</tr>
<tr>
<td>µL 2,4-D</td>
<td>50</td>
</tr>
<tr>
<td>mL myo-inositol</td>
<td>1</td>
</tr>
</tbody>
</table>
Since initial phosphate concentrations are different in the MS-medium for NT-1 cells, two different stock solutions had to be prepared as shown in Table 2-2:

**Table 2-2: Stock solutions for the preparation of the NT-1 MS-medium**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration stock solution [mM]</th>
<th>Final concentration [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PbMo stock solution (100x)</td>
<td>KH$_2$PO$_4$</td>
<td>124.9</td>
</tr>
<tr>
<td></td>
<td>H$_3$BO$_3$</td>
<td>10.03</td>
</tr>
<tr>
<td></td>
<td>Na$_2$MoO$_4$.2H$_2$O</td>
<td>0.1</td>
</tr>
<tr>
<td>Phosphate stock solution I</td>
<td>K$_2$HPO$_4$</td>
<td>78.65</td>
</tr>
</tbody>
</table>

**For 1 L self-made MS-medium for NT-1 cells:**

- 10 mL of each stock solution (halogen, sulfate, NaFe-EDTA and nitrate)
- 10 mL of PbMo stock solution (Table 2-2)
- 10 mL of phosphate stock solution I
- 30 g/L sucrose
- 0.5 g/L MES
- 250 µL Thiamine-HCl
- 1110 µL 2,4-D
- 1 mL myo-inositol

The solutions were filled up to 1 L with deionized water. Before autoclaving (121°C, 21 min), the pH was adjusted to 5.8 with 1 M KOH in both media.

**2.3.2 Media modification for BY-2 cells**

The media modification was focused on the nitrogen source of the MS-medium. The amount of nitrate and ammonium as the sole nitrogen sources in the MS-medium and the amount of the initial amount of ammonium were changed. It is important to note, that all other media components had the same concentration as in the commercial MS-medium. Thus, differences in cell growth as well as differences in the protein production can only be attributed to the different nitrogen source or its amounts.

In total three different MS-media for BY-2 cells were created:

**reduced initial NH$_4^+$ (10 mM ammonium)**

- 10 mL of each stock solution (halogen, sulfate, PbMo, and NaFe-EDTA)
- 4.75 mL (2.06 M NH$_4$NO$_3$ stock solution)
- 14.55 mL (1.98 M KNO$_3$ stock solution)
- 30 g/L sucrose
- 150 µL Thiamine-HCl
- 50 µL 2,4-D
- 1 mL myo-inositol
only KNO₃
10 mL of each stock solution (halogen, sulfate, PbMo, and NaFe-EDTA)
6.068 g/L KNO₃
30 g/L sucrose
150 µL Thiamine-HCl
50 µL 2,4-D
1 mL myo-inositol

increased initial NH₄⁺ (30 mM ammonium)
10 mL of each stock solution (halogen, sulfate, PbMo, and NaFe-EDTA)
14.75 mL (2.06 M NH₄NO₃ stock solution)
4.55 mL (1.98 M KNO₃ stock solution)
30 g/L sucrose
150 µL Thiamine-HCl
50 µL 2,4-D
1 mL myo-inositol

2.3.3 Media modification for NT-1 cells
For NT-1 cells, the influence of two different macro nutrients was investigated: nitrogen and phosphate. Hence, three different stock solutions were prepared as shown in Table 2-3:

Table 2-3: Stock solutions necessary for the medium modification

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Concentration stock solution [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium nitrate stock solution (100x)</td>
<td>3092.21</td>
</tr>
<tr>
<td>Potassium nitrate stock solution (100x)</td>
<td>3758.66</td>
</tr>
<tr>
<td>Phosphate stock solution II</td>
<td>1.02</td>
</tr>
</tbody>
</table>

The influence of the initial phosphate concentration was examined using three different initial phosphate concentrations: 1.25 mM, 2.04 mM and 4.08 mM:

For 1 L modified MS-medium for NT-1 cells containing 1.25 mM phosphate:

10 mL of each stock solution (halogen, sulfate, NaFe-EDTA and nitrate)
10 mL of PbMo stock solution (Table 2-2)
30 g/L sucrose
0.5 g/L MES
250 µL Thiamine-HCl
1110 µL 2,4-D
1 mL myo-inositol

b) containing 2.04 mM phosphate: + 10 mL phosphate solution I (Table 2-1)
c) containing 4.08 mM phosphate: + 10 mL phosphate solution I + 20 mL phosphate solution II (Table 2-2)

Before autoclaving (121°C, 21 min), the pH was adjusted to 5.8 with 1 M KOH in all media.

The influence of the initial nitrogen source was investigated in two different ways:

1) Variation of the nitrate to ammonium ratio with at same amount of total nitrogen (60 mM)
2) Increase of the total amount of nitrogen in the MS-medium
The commercial MS-medium contains 20.61 mM ammonium and 39.4 mM nitrate (simplified as a ratio of 20:40). Moreover, two different MS-media with ratios of 10:50 and 30:30 were created. The total initial amount of the concentrations of ammonium, nitrate and potassium are shown in Table 2-4.

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Molar ratio of ammonium to nitrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20:40</td>
</tr>
<tr>
<td>Ammonium</td>
<td>20.61</td>
</tr>
<tr>
<td>Nitrate</td>
<td>39.4</td>
</tr>
<tr>
<td>Potassium</td>
<td>24.45</td>
</tr>
</tbody>
</table>

For 1 L modified MS-medium for NT-1 cells with different nitrogen ratios:

- 10 mL of each stock solution (halogen, sulfate and NaFe-EDTA)
- 10 mL of PbMo stock solution (Table 2-2)
- 20 mL of phosphate solution II (Table 2-3)
- 30 g/L sucrose
- 0.5 g/L MES
- 250 μL Thiamine-HCl
- 1110 μL 2,4-D
- 1 mL myo-inositol

- a) 10 mL nitrate stock (ratio 20:40)
- b) 10 mL ammonium nitrate stock (ratio 30:30)
- c) 1.3 mL ammonium nitrate stock + 10 mL potassium nitrate stock (ratio 10:50)

In a further experiment, the influence of the total initial nitrogen concentration on NT-1 cell growth and on the protein concentration was investigated. Table 2-5 gives an overview of the different concepts of experiments with their respective initial concentrations of ammonium, nitrate and potassium.

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Molar ratio of ammonium to nitrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30:40</td>
</tr>
<tr>
<td>Total nitrogen</td>
<td>70</td>
</tr>
<tr>
<td>Ammonium</td>
<td>30</td>
</tr>
<tr>
<td>Nitrate</td>
<td>40</td>
</tr>
<tr>
<td>Potassium</td>
<td>15.65</td>
</tr>
</tbody>
</table>
For 1 L modified MS-medium for NT-1 cells containing different initial nitrogen concentrations:

- 10 mL of each stock solution (halogen, sulfate and NaFe-EDTA)
- 10 mL of PbMo stock solution (Table 2-2)
- 20 mL of phosphate solution II
- 30 g/L sucrose
- 0.5 g/L MES
- 250 µL Thiamine-HCl
- 1110 µL 2,4-D
- 1 mL myo-inositol

a) 10 mL nitrate stock + 3.3 mL potassium nitrate stock (ratio 30:40)
b) 10 mL ammonium nitrate stock + 5 mL potassium nitrate stock (ratio 30:50)
c) 13.3 mL ammonium nitrate stock (ratio 40:40)

### 2.3.4 Influence of mannitol as an inert osmolyte

Mannitol was used as an inert osmolyte to increase artificially the osmotic pressure of the MS-medium. *N. tabacum* cells were cultivated in the RAMOS device and shake flasks in parallel (180 rpm, 26°C, 5 cm shaking diameter). After 101 h 4 mL of a 1.35 M sterile mannitol stock solution was added to the shake flasks with 50 mL filling volume. Added mannitol corresponds to a final osmolality of 0.1 Osm/kg in the MS-medium. As a reference 4 mL sterile deionized water instead of mannitol was added.

### 2.4 Cultivation of *Nicotiana tabacum* suspension cells

#### 2.4.1 Subcultivation of cell suspensions

Subcultivation of *N. tabacum* cells was performed weekly by pipetting 2% for wild type and 5% for transgenic cells (v/v) from a 7-day old culture in 50 mL MS-medium in 250 mL shake flasks. Finally, cells were cultivated in the dark at 180 rpm and 26°C with a 5 cm shaking diameter.

#### 2.4.2 Cultivation conditions of cell suspensions

In order to identify the optimal growth conditions for tobacco suspension cells, tobacco cells were cultivated under two different cultivation conditions. Under IME conditions, cells were cultivated in MS-medium in narrow-neck Erlenmeyer flasks at 26°C in the dark on a rotary shaker operating at 180 rpm with a shaking diameter of 5 cm. Under Dow AgroSciences conditions (DAS), however, cells were cultivated in narrow-neck Erlenmeyer flasks at 25°C and 130 rpm with a shaking diameter of 5 cm. Unless otherwise stated, IME cultivations were used for the cultivation.
2.5 Online measurement of the oxygen transfer rate (OTR)

For the online measurement of the OTR in shake flasks, the RAMOS device was used, described by Anderlei and Büchs (2001) and Anderlei et al. (2004). Measuring the OTR online during cultivation is the most suitable way to quantify the physiological state of aerobic microorganisms and cell cultures as all steps in the aerobic metabolism necessitates oxygen. The RAMOS-flasks are modified 250 mL Erlenmeyer flasks with 4 different cup holders as illustrated in Figure 2-1A.

![Figure 2-1: Images of a 250 mL RAMOS flask (A) in comparison to an ordinary 250 mL Erlenmeyer flask (B).](image)

The bracket for the oxygen sensor is on the top of the flask. Two cup holders serve for the gassing and the last one is for the inoculation of the flask. These RAMOS-flasks are characterized by identical hydrodynamic and gas phase conditions as commercial Erlenmeyer flasks covered with a cotton plug (Anderlei et al. 2004) (Figure 2-1B). It should be mentioned that the measuring principle is non-invasive. Thus, identical hydrodynamic conditions are assured.

![Figure 2-2: Structure of the RAMOS device (Anderlei and Büchs 2001).](image)
The structure of the RAMOS device is shown in Figure 2-2. Cotton acts as a sterile barrier in the inlet and outlet stub to avoid contaminations in the RAMOS flask. Throughout this work a flow rate of 10 mL/min was used. The process control unit than calculates the OTR and CTR.

The measuring principle of the RAMOS device is quasi-continuous and splitted into two different phases: a measuring phase and a rinsing phase (Figure 2-3).

![Figure 2-3: Partial pressure of oxygen (solid) and carbon dioxide (dashed) during a measuring cycle in the RAMOS device. I rinsing phase, II measuring phase, X sensor calibration (ANDERLEI AND BÜCHS 2001).](image)

During the rinsing phase (I), the valves are open and air is flushed through the flasks. Prior the measuring phase (II), the oxygen sensors are calibrated at a constant oxygen partial pressure (indicated by X in Figure 2-3). In the measuring phase, the valves are closed and due to the respiration activity of the microorganisms the partial pressure of oxygen in the head-space decreases. The OTR is calculated automatically from the change of the partial pressure according to the following equation:

$$\text{OTR} = \frac{n_{O_2}}{V_L \cdot \Delta t} = \frac{\Delta p_{O_2}}{R \cdot T \cdot V_L}$$

Eq.: 2-1

with

- $O\text{TR}$ oxygen transfer rate [mol/L/h]
- $n_{O_2}$ moles of oxygen [mol]
- $V_L$ liquid volume [L]
- $\Delta p_{O_2}$ difference of oxygen partial pressure [bar]
- $\Delta t$ time of the measuring phase [h]
- $V_G$ gas volume [L]
- $R$ universal gas constant [bar · L/mol · K]

With this technique it is possible to gather useful information about the cultivation conditions and the physiology of the organisms in up to eight individual RAMOS flasks in parallel (ANDERLEI ET AL. 2004).
Tobacco cells were cultivated in parallel in modified 250 mL RAMOS shake flasks. The shaker was operated in the dark at 180 rpm, 5 cm shaking diameter and at 26°C. All flasks had a filling volume of 50 mL. In the case offline samples were needed, conventional non-monitored 250 mL Erlenmeyer flasks were used in addition to RAMOS flasks, inoculated by the same tobacco cell master mix. One flask was used for each sample in order to avoid a modification of the culture conditions due to the removed sample volume.

2.6 Analytical methods

2.6.1 Offline measurement of packed cell volume, fresh and dry weight
The cell growth of tobacco suspension cells was monitored by determining the packed cell volume (PCV), the fresh and dry weight. For the determination of the PCV 10 ml of cell suspension was centrifuged (4300 g, 5 min) in a 15 ml falcon tube and finally the PCV was read off on the falcon tube. Fresh weight was analyzed by vacuum-filtrating 10 mL of cell suspension by using a pre-weighed Whatman filter (No. 3, 55 mm diameter). The resulting biomass pellet was weighed (wet weight) and stored at 105°C in an oven until the mass, weighed with an electronic precision balance (SBC 31, Scaltec, Göttingen), remained constant (dry weight).

2.6.2 Determination of different sugars
Sugar analysis was performed via high-performance liquid chromatography (HPLC). A carbohydrate column Pb2+ (No. 52898230, CS-Chromatography, Langerwehe) was used to separate the different constituents of a sample. The detection was carried out by a RI-Detector (Shodex, Tokyo, Japan), and for the sample analysis, the software Chromelion (Dionex, Idstein) was used.

2.6.3 Determination of the osmolality
The osmolality, measured via cryoscopy, was detected using the Osmomat 030 (Gonotec, Berlin). After a 2-point calibration, the supernatant was analyzed according to the manufacturer’s protocol.

2.6.4 Determination of the conductivity and pH-value
Both, the conductivity and pH-value were measured directly in the tobacco cell culture broth. The conductivity was determined using the digital conductivity meter LF 340-A (WTW, Weilheim), and the pH-value was measured using the pH-Meter pH510 (Eutech Instruments, Simi Valley, USA).
2.6.5 Photometric measurement of phosphate, ammonium, nitrate and sulfate

The phosphate concentration of the supernatant was measured using a round cuvette test kit (No. 1.00616.0001, Merck, Darmstadt). This assay is based on the detection of phosphomolybdenum blue (PMB) that is formed in the presence of phosphate ions, analogous to DIN EN 1189 D11. The ammonium concentration was quantified using a different round cuvette test (No. 1.14559.0001, Merck). Here, a blue indophenol derivate was detected analogous to DIN 38406 E5. The concentration of nitrate ions (No. 1.14883.0001, Merck), based on DIN 38405 D9, was determined by measuring the 4-nitro-2.6-dimethylphenol. All three ion concentrations were measured using the device Nova Spectroquant 60 (Merck).

2.6.6 Measurement of fluorescent proteins

Within this work two different fluorescent proteins have been used: GFP and YFP. Both measurements were performed offline in 48-well micro titer plates (MTPs) (Costar, flat bottom, clear). YFP exhibits an excitation maximum at 525 nm, an emission maximum at 538 nm and it was detected via 2D fluorescence measurements (Fluoromax 4P; HORIBA JobinYvon, Unterhaching). Therefore, 0.125 g wet weight of plant cells was resuspended in 500 µL supernatant and the MTP was shaken at 500 rpm. Settings for the 2D fluorescence measurement were as follows: Excitation 500 – 550 nm, increment 1 nm, slit 5 nm. Emission: 513 – 563 nm, increment 1 nm, slit 5 nm. In both cases, the integration time was 0.1 s. As a reference, the fluorescence of 0.125 g wet weight of wild type cells was measured in parallel and subtracted from the values of the transgenic cell line. The detection of GFP was performed in the BioLector. Here, 0.1 g wet weight was resuspended in 500 µL supernatant and shaken at 500 rpm. For the determination of the volumetric fluorescence 500 µL culture broth was used instead. The excitation was 485 nm and the emission 520 nm at gain 30. As a reference, 0.1 g wild type cells were measured under identical conditions.

2.6.7 Protein analysis

2.6.7.1 Extraction of total soluble protein

Tobacco suspension cells were separated from the medium by filtration, and 0.1 g cells were resuspended in 0.6 ml extraction buffer and stored at -20°C. The cells were thawed and destroyed via ultrasonication (60 s, 55 W, duty cycle 0.7) on ice (Labsonic U, B. Braun, Melsungen). Cell debris was removed by centrifugation (10 min, 14,000 rpm, 4°C) and the supernatant, containing the soluble proteins, was used for further analysis.
Material and Methods

Extraction buffer, pH 6.0

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>5 mM</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>5 mM</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>10 mM</td>
</tr>
</tbody>
</table>

in PBS stored at 4°C

2.6.7.2 Quantification of total protein amount

The protein concentration was quantified using the Bradford assay (Bradford 1976). The respective concentrations were measured according to the manufacturer’s microtiter plate protocol (Bio-Rad Laboratories, Munich) by using bovine serum albumin (BSA) as standard. Data were evaluated applying the software Origin 8.5 according to the manufacturer’s protocol.

2.6.7.3 SDS-polyacrylamide gel electrophoresis

Discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gels (Laemmli 1970) were used for separating protein samples. Therefore, the XCell SureLock mini-cell electrophoresis system was used (Invitrogen, Carlsbad, CA, USA). Two micrograms protein were incubated with 2 µL of five-fold concentrated loading buffer in a total volume of 10 µL at 99°C for 10 min. NuPage® 4-12% (w/v) Bis-Tris gels (Invitrogen) were prepared according to the manufacturer’s protocol, and the prestained Fermentas PageRuler® marker (Fermentas, St. Leon-Rot) was used as a molecular mass standard. Pure GFP (Evrogen, Moscow, RUS) and the supernatant of the wild type BY-2 strain were used as positive and negative controls, respectively. Visualization of separated proteins was performed using Coomassie G-250, SimplyBlue SafeStain (Invitrogen) solution for 1 h at room temperature. For decolorization the gel was stored in deionized water over night at room temperature in the dark.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mL loading buffer (5x), pH 6.8</td>
<td></td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>1.25 mL</td>
</tr>
<tr>
<td>SDS</td>
<td>1 g</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>5.2 mL</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>Glycerol</td>
<td>3 mL</td>
</tr>
<tr>
<td>500 mL SDS Running buffer (20x), pH 8.8</td>
<td></td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>60.6 g</td>
</tr>
<tr>
<td>SDS</td>
<td>10 g</td>
</tr>
<tr>
<td>MES</td>
<td>97.6 g</td>
</tr>
<tr>
<td>EDTA</td>
<td>3 g</td>
</tr>
</tbody>
</table>

2.6.7.4 Western blot analysis

For Western blot analysis the SDS-polyacrylamide gels were transferred onto a nitrocellulose membrane (Whatman, Springfield Mill, UK) by using the XCell II Blot module (Invitrogen) (25 V constant, 60 min). The membranes were blocked at room temperature in milk powder solution for 30 min. Subsequently, the membranes were incubated at 4°C over night with a rabbit polyclonal antibody against GFP (Evrogen, Moscow, RUS) diluted 1:20,000 in phosphate buffered saline (PBS) to detect GFP. After the membrane was washed thrice with PBST (PBS containing 0.05% Tween 20), the membranes were then incubated with alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, Suffolk, UK)
diluted 1:5,000 in PBS at room temperature for 1 h. Finally, bound antibodies were visualized by incubating the membrane for 10 min with nitro blue tetrazolium chloride/5-bromo 4-chloroindol-3-yl phosphate (NBT-BCIP) diluted 1:100 in AP-buffer. The Western blot was analyzed densitometrically using the scanner ‘Perfection V700’ (Epson, Suwa, Japan). Band intensities were quantified using the software TotalLab TL100 (Nonlinear Dynamics, Newcastle, UK).

<table>
<thead>
<tr>
<th>Phosphate buffered saline (PBS) buffer (10x), pH 7.4</th>
<th>PBST buffer, pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl 1.37 M</td>
<td>Tween 20 0.05% (v/v)</td>
</tr>
<tr>
<td>KCl 27 mM</td>
<td>in PBS</td>
</tr>
<tr>
<td>Na₂HPO₄ · 12 H₂O 81 mM</td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄ 15 mM</td>
<td></td>
</tr>
</tbody>
</table>

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<td>NaCl 1.37 M</td>
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<td>KCl 27 mM</td>
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</tr>
<tr>
<td>Na₂HPO₄ · 12 H₂O 81 mM</td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄ 15 mM</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Milk powder solution</th>
<th>Transfer buffer, pH 9.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk powder 5% (w/v)</td>
<td>Tris-HCl 192 mM</td>
</tr>
<tr>
<td>in PBST</td>
<td>Glycine 25 mM</td>
</tr>
<tr>
<td></td>
<td>Methanol 20 % (v/v)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Alkaline phosphatase buffer (AP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl 100 mM</td>
</tr>
<tr>
<td>NaCl 100 mM</td>
</tr>
<tr>
<td>MgCl₂ 5 mM</td>
</tr>
</tbody>
</table>

2.6.7.5 “Enzyme-linked Immunosorbent Assay” ELISA

ELISA (CLARK AND ADAMS 1977) analysis was used for quantification of plant-expressed HA. F-96 MaxiSorp microtiter plates (Nunc, Wiesbaden) were first coated with chicken anti-hemagglutinin (HA) antibodies diluted in PBS buffer over night at 4°C. Free-binding sites were subsequently blocked with 3% (w/v) BSA dissolved in PBS. All following incubation steps were performed at room temperature with shaking. A serial dilution of HA-standard from 100 to 1.5625 ng/mL as well as a serial dilution from the samples was applied to the wells. Then, the plates were incubated for 60 min. Afterwards, those plates were incubated with murine anti-HA mAb 9E7-37-37 (Benchmark Biolabs, Lincoln, NE, USA) diluted 1:2,000 in PBST for 60 min., followed by an incubation with goat anti-mouse (No. 31437, ThermoScientific, Rockford, IL, USA) polyclonal antibody diluted 1:5,000 in PBST for 60 min for the detection of the bound primary antibody. Bound secondary antibodies were detected by incubation with the TMB ELISA substrate (Pierce No. 34028, ThermoScientific) for 30 min. The reaction was stopped using 1 N sulfuric acid. Between the different incubations steps, plates were washed thrice with PBST. Subsequently, the absorbance at 405 nm was determined using the Synergy 4 microtiter plate reader (BioTek Inst.,Winooski, VT, USA) and the binding data was evaluated using Origin 8.5 by determining the slope in the linear range of the dose-response curve by linear regression.
2.7 Characterization of the maximum oxygen transfer capacity using the sulfite system

To simulate an oxygen consumer and to characterize the maximum oxygen transfer capacity (OTR\textsubscript{max}) at different cultivation conditions, the sulfite system was used as a chemical model system (Hermann et al. 2001). The test solution is composed of 0.012 M Na\textsubscript{2}HPO\textsubscript{4}/NaH\textsubscript{2}PO\textsubscript{4} phosphate buffer, 1 M Na\textsubscript{2}SO\textsubscript{3}, and the catalyst CoSO\textsubscript{4} in 10\textsuperscript{-7} M. The pH value is adjusted to pH 8 with sulfurous acid prior to the experiments. The stoichiometric oxidation of sulfite ions is as follows:

\[
\text{HSO}_3^- + \frac{1}{2} O_2 \xrightarrow{\text{Catalyst}} \text{SO}_4^{2-} + H^+ \quad \text{Eq.: 2-2}
\]

During the reaction, the pH declines to values between 4 and 5 since hydrogen sulfate is completely dissociated. With the required duration for the sulfite oxidation \((t_{\text{OX}})\), the initial concentration of sulfite \((c_{\text{sulfite}} = 1 \text{ mol/L})\) and the stoichiometric coefficient for oxygen \(v_{O_2} = 0.5\), the maximum oxygen transfer rate (OTR\textsubscript{max}) to the solution can be calculated according the following equation:

\[
\text{OTR}_{\text{max}} = \frac{c_{\text{sulfite}} \cdot v_{O_2}}{t_{\text{OX}}} \quad \text{Eq.: 2-3}
\]

In this work, the sulfite system is used to characterize the maximum oxygen transfer capacity for IME as well as for DAS cultivation conditions (2.4.2).

2.8 Polymer based controlled-release systems in plant suspension culture

By using a diffusive slow-release technique, a fed-batch mode in shaken bioreactors can be realized. This technique was introduced by Jeude et al. (2006) and has already been successfully employed by Sтокmann et al. (2009) for a controlled-release of glucose in E. coli cultivations. In this work, ammonium chloride instead of glucose was embedded in a silicone elastomer disc. The release of ammonium chloride under fermentation conditions was studied by adding one ammonium chloride containing silicone elastomer disc (diameter \(D = 25 \text{ mm}\); height \(H = 2 \text{ mm}\)) into the culture broth. In total 20 mmol ammonium chloride was released in a period of 140 h according to a square root of time kinetic. Kinetic experiments of the ammonium release were performed by Dr. Barbara Dittrich at the Institute for Technical and Macromolecular Chemistry (ITMC), RWTH Aachen. Contamination of the culture was prevented by washing the discs in pure ethanol and after cleaning in sterile deionized water the discs were added into the culture. To guarantee equal initial cell concentrations in the
RAMOS flasks, a master mix of cells was prepared and distributed to the RAMOS flasks. One elastomer disc was added in each shake flask. For the ammonium-enriched medium, sterile ammonium chloride was added into the medium to a final concentration of 0.04 mol/L from a sterile 1 M stock solution.

![Figure 2-4: A: Cut and top-view of a silicone elastomer disc with enclosed nutrient crystals. B: Shake flask filled with culture broth and four silicone elastomer discs as polymer-based release systems (JEUDE ET AL. 2006).](image)

2.9 Cultivation of BY-2 cells in the BioLector

Cultivations in MTPs were performed with a commercial BioLector device (Figure 2-5B, m2p-labs GmbH, Baesweiler). The prototype of the device is shown in Figure 2-5A and was already described by SAMORSKI ET AL. (2005) and KENSY ET AL. (2009). The Nicotiana tabacum BY-2 Cv MYGFP-KDEL cell line was used to investigate plant cell growth and the influence of different medium compositions on GFP formation. Compositions of the various modified MS-media are described in section 2.3.1 of this chapter. All experiments were carried out in sterile black 48-well MTP with round wells (MTP-R48-B, m2p-labs GmbH) with a total filling volume of 1000 µL which were sealed with two gas-permeable sealing films (F-GP-10, m2p-labs GmbH) and one perforated sealing film (F-R48-10, m2p-labs GmbH) to avoid evaporation. In addition, humidified air was continuously flushed through the measuring chamber. The cultivation conditions were as follows: 26°C, 3 mm shaking diameter, 900 rpm shaking frequency. Plant cell growth was monitored via scattered light intensity which was detected at an excitation wavelength of 620 nm. The GFP fluorescence was detected at an excitation wavelength of 485 nm and an emission wavelength 520 nm. To eliminate factors that might influence the scattered light intensity (I) such as the type of MTP, possible noise of the media or geometrical positioning the experimental data
were standardized (SAMORSKI ET AL. 2005). Hence, the initial value of scattered light intensity \( I_0 \) was subtracted from every value \( (I-I_0) \). The same was done for the fluorescence signal to detect the GFP fluorescence.

![Figure 2-5: A: Scheme of the BioLector; B: commercial BioLector (photo by m2p-labs).](image)

### 2.10 Fermentation of *Nicotiana tabacum* BY-2 cells

For the fermentation experiments, the transgenic tobacco cell line *N. tabacum* M12TAD was used. To compare plant cell growth at different scales, experiments were performed in shake flasks, in RAMOS and in a fermenter at the same time. Fermentations were performed in a 2 L BIOSTAT®Bplus fermenter (Sartorius AG, Göttingen). The geometric dimensions are summarized in Table 2-6.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Dimension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermenter volume</td>
<td>( V_R )</td>
<td>2 L</td>
</tr>
<tr>
<td>Filling volume</td>
<td>( V_L )</td>
<td>1.5 L</td>
</tr>
<tr>
<td>Fermenter height</td>
<td>( H_R )</td>
<td>0.24 m</td>
</tr>
<tr>
<td>Fermenter diameter</td>
<td>( D_R )</td>
<td>0.127 m</td>
</tr>
<tr>
<td>Number of stirrer</td>
<td>( n_{st} )</td>
<td>1</td>
</tr>
<tr>
<td>Bottom clearance of 1(^{st}) stirrer</td>
<td>( L_1 )</td>
<td>0.048 m</td>
</tr>
<tr>
<td>Number of stirrer blades</td>
<td>( n_{bl} )</td>
<td>6</td>
</tr>
<tr>
<td>Height of an agitator blade</td>
<td>( h )</td>
<td>0.0106 m</td>
</tr>
<tr>
<td>Blade diameter</td>
<td>( d_R )</td>
<td>0.053 m</td>
</tr>
</tbody>
</table>

Prior the fermentations, the pH-Meter was calibrated by a two-point calibration. For the analysis of the exhaust gas, the exhaust gas analyzer NGA 2000 was used which was calibrated with nitrogen (zero) and test gas (25% \( \text{O}_2 \), 5% \( \text{CO}_2 \)). The fermenter was sterilized containing 50 mL deionized water at 121°C for 21 min. To assure identical initial cell concentrations, a 2 L inoculation flask containing 2 L commercial MS-medium (2.3) was sterilized (121°C, 21 min) and inoculated with 5% BY-2 M12TAD cells from a 7-day old
culture. First, shake flasks and RAMOS flasks without antifoam were filled. Second, antifoam (Pluronic® L61, BASF, Ludwigshafen) was added to the inoculation flask with a final concentration of 0.1 g/L, and further Erlenmeyer flasks and the fermenter were filled with the inoculation flask. To ensure a sufficient mixing the 2 L inoculation flask was put on a magnetic stirrer during the inoculation procedure. The Erlenmeyer flasks as well as the RAMOS flasks were cultivated under IME conditions (2.4.2). To assure a constant power input the fermentation was performed with a single-stage agitator with an agitation rate \( n_R = 357 \text{ rpm} \) and an aeration rate \( q_{\text{in}} \) of 0.25 vvm. In all fermentation experiments the temperature was constant at 26°C.

### 2.10.1 Calculation of the OTR and CTR during fermentation

During fermentations, the oxygen transfer rate can be calculated according the following equation:

\[
OTR = \frac{60 \cdot Q_G}{V_{\text{mol}} \cdot V_L} \cdot \left( y_{O_2,\text{in}} - \left( \frac{1 - y_{O_2,\text{out}} - y_{CO_2,\text{in}}}{1 - y_{O_2,\text{out}} - y_{CO_2,\text{out}}} \right) \cdot y_{O_2,\text{out}} \right)
\]

Eq.: 2-4

The carbon dioxide transfer rate describes the amount of carbon dioxide that is stripped from the liquid phase to the gaseous phase during a certain period of time and can be calculated according the following equation:

\[
CTR = \frac{60 \cdot Q_G}{V_{\text{mol}} \cdot V_L} \cdot \left( y_{CO_2,\text{out}} - \left( \frac{1 - y_{O_2,\text{in}} - y_{CO_2,\text{in}}}{1 - y_{O_2,\text{out}} - y_{CO_2,\text{out}}} \right) - y_{CO_2,\text{in}} \right)
\]

Eq.: 2-5

with

- **OTR** oxygen transfer rate \([\text{mmol/L/h}]\)
- **CTR** carbon dioxide transfer rate \([\text{mmol/L/h}]\)
- **\(Q_G\)** volumetric gas flow rate \([\text{L/min}]\)
- **\(V_{\text{mol}}\)** molar gas volume \([\text{L/mol}]\)
- **\(V_L\)** filling volume \([\text{L}]\)
- **\(y_{O_2,\text{in}}\)** oxygen mole fraction of the supply air \([\text{mol/mol}]\)
  (with air: 0.2095%)
- **\(y_{O_2,\text{out}}\)** oxygen mole fraction of exhaust gas \([\text{mol/mol}]\)
- **\(y_{CO_2,\text{in}}\)** carbon dioxide mole fraction of inlet gas \([\text{mol/mol}]\)
  (with air: 0.035%)
- **\(y_{CO_2,\text{out}}\)** carbon dioxide mole fraction of exhaust gas \([\text{mol/mol}]\)
2.11 Power input shaker

The volumetric power input in shake flasks was determined by using the power input shaker (BÜCHS ET AL. 2000A). The experimental conditions corresponded to the IME cultivation conditions (2.4.2). Prior the experiment, a reference measurement with 14 empty Erlenmeyer flasks was performed in order to determine the air resistance and the mechanical friction losses of the shaker drive. During the reference measurement the weight of the liquid was evened with metal weights.

![Figure 2-6: Measuring device for the determination of the power consumption in shaking flasks: (1) drive with integrated torque sensor; (2) free wheel; (3) flexible coupling; (4) rotary table shaker; (5) speed measurement and control; and (7) data acquisition (BÜCHS ET AL. 2000A).](image)

2.12 Viscosity measurements

The dynamic viscosity $\eta$ was measured with the Physica MCR 301 Modular Compact Rheometer (Anton Paar GmbH, Graz, AUT) using the parallel & plate (serial number: 15847) and the coaxial cylinder (serial number: 13220) measuring system at 26°C. Depending on the age of the culture and its viscosity the parallel & plate measuring system (0-3 days) or the coaxial cylinder measuring system (4-8 days) was used. Using the parallel & plate measuring system a gap width of 0.6 mm and a sample volume of 1.6 mL was used. For the coaxial cylinder measuring system, a sample volume of 21 mL was filled in the cylinder. The dependency of the viscosity on the shear rate was described by the Ostwald-de Waele relationship:

$$\eta = K \cdot \gamma^{m-1} \quad \text{Eq.: 2-6}$$

with

- $\eta$: dynamic viscosity [Pa·s]
- $K$: flow consistency index [Pa·s$^m$]
- $\gamma$: shear rate [1/s]
m flow behavior index [-]

The parameter $K$ and $m$ were estimated according to HENZLER AND SCHÄFER (1987). For the calculation of the effective shear rate $\gamma_{\text{eff}}$ and the effective viscosity $\eta_{\text{eff}}$ in shake flasks the following equations were used (PETER 2006):

$$\frac{\gamma_{\text{eff}}}{n_s} = 8.42 \cdot \left( \frac{d}{V_L^{1/3}} \right)^{-0.25} \rho^{0.44} \cdot n_s^{0.44} \cdot d^{0.88} \cdot n_{\text{eff}}^{-0.44}$$  \hspace{1cm} \text{Eq.: 2-7}

$$\eta_{\text{eff}} = \left[ 8.42 \cdot d^{0.63} \cdot V_L^{0.08} \cdot \rho^{0.44} \cdot n_s^{1.44} \cdot K^{\frac{1}{m+1}} \right] \left( \frac{m-1}{0.44m+0.56} \right)$$  \hspace{1cm} \text{Eq.: 2-8}

with $\eta_{\text{eff}}$ effective viscosity [Pa · s]  
$d$ inner diameter shake flask [m]  
$V_L$ filling volume [m³]  
$\rho$ density [kg/m³]  
$n_s$ shaking frequency [1/s]  
$K$ flow consistency index [Pa·s$^m$]  
$m$ flow behavior index [-]  
$\gamma_{\text{eff}}$ effective shear rate [1/s]

To calculate the apparent viscosity in a stirred tank reactor, the average shear rate as function of the operating conditions of the reactor is required. Since the shear rate in a fermenter is not constant, a mean effective shear rate ($\gamma_{\text{rep}}$) according Metzner and Otto was calculated (KRAUME 2003):

$$\gamma_{\text{rep}} = k \cdot n_R$$  \hspace{1cm} \text{Eq.: 2-9}

with $\gamma_{\text{rep}}$ representative shear rate [1/s]  
$k$ Metzner-Otto constant [-]  
$n_R$ agitation rate [1/s]

For the Rushton turbine the Metzner-Otto constant is 11 (KRAUME 2003). Intrinsically, the Metzner-Otto concept has been developed for the laminar flow regime in a fermenter but has also been extrapolated into the turbulent flow regime.
Chapter 3  Fundamentals of plant cell growth using RAMOS

3.1 Different transgenic cell lines applied within this thesis

Various transgenic cell lines have been established from two wild type cell lines by the cooperation partner Fraunhofer IME (Figure 3-1). These wild type cell lines of NT-1 and BY-2 cells originate from Dow AgroSciences (Indianapolis, USA). Suspension cultures of the transgenic tobacco cell lines expressing different fluorescent marker proteins have been developed from these tobacco wild type strains. Figure 3-1 provides a survey of all transgenic cell lines developed by the cooperation partners.

![Diagram of cell lines](image)

Figure 3-1: Different cell lines applied within this thesis. Red boxes indicate that results obtained with these cell lines are presented in this thesis.

With the BY-2_{CsVMV}GFP-KDEL and the NT-1_{CsVMV}HA cell line, the majority of the experiments were performed (highlighted by the thick red boxes, Figure 3-1). In total three different promoters were tested. An actin promoter originated from *Arabidopsis thaliana* (AtAct); an actin promoter originated from *Cucumis melo* (CmAct) and the *Cassava vein mosaic virus* promoter (CsVMV). Moreover, two transgenic BY-2 cell lines contained the
leader sequence “KDEL” which encodes retention of the expressed GFP at the endoplasmatic reticulum of the cell (MUNRO AND PELHAM, 1987).

Based on the fluorescence intensity, these promoters showed significant differences in the expression level. The viral CsVMV promoter clearly showed the highest level of expression (data not shown) which is in agreement with findings of other researchers (VERDAUGER ET AL. 1998). Hence, and also due to molecular biological findings of the cooperation partner Fraunhofer IME (i.e. regeneration of protoplasts), the BY-2CsVMV-GFP-KDEL cell line was chosen as the target cell line for investigating the recombinant protein production.

3.2 Growth of different wild type *Nicotiana tabacum* suspension cells

Two different tobacco suspension cell lines were applied in this work: BY-2 and NT-1. The NT-1 cell line is mentioned as the sibling of the BY-2 cell line and should be genetically identical to the BY-2 cell line (NAGATA ET AL. 1992). NT-1 cells were separated from the BY-2 cells in 1982 and are being maintained in several laboratories, mainly in North America (NAGATA AND KUMAGAI 1999). For the first time, these tobacco cell lines were cultivated in the RAMOS device (Figure 3-2A). A suspension culture, established from the neem tree, *Azadirachta indica* has already been cultivated and successfully characterized in the RAMOS device (RAVAL ET AL. 2003).

Figure 3-2A presents the oxygen transfer rates (OTRs) of three different tobacco wild type cell lines during cultivation. As carbon dioxide transfer rate values were similar to those of the oxygen transfer rate (data not shown), only oxygen transfer rates are shown in Figure 3-2A. The OTR-curves are presented in duplicates with a standard error less than ±2%. This confirms that the RAMOS device can precisely measure OTR-values lower than 1 mmol/L/h. The inoculation density for the different wild type cell lines was 2%. All cell lines were cultivated in the commercial MS-medium for BY-2 and NT-1 cells (2.3), respectively.
Figure 3-2: Cultivation of *Nicotiana tabacum* wild type cells in MS-medium and *E. coli* in TB-medium. (A) Oxygen transfer rates (OTR) of three different *Nicotiana tabacum* wild type cells in MS-medium. Maximum growth rates: BY-2\(_{\text{IME}}\) = 0.04 l/h; BY-2\(_{\text{DAS}}\) = 0.038 l/h and NT-1\(_{\text{DAS}}\) = 0.029 l/h (B) oxygen transfer rates of a non-oxygen limited growth of *E. coli* K12 and BY-2 wild type cells. Experimental conditions for BY-2 cells: Flask volume 250 mL, filling volume 50 mL, MS-medium, initial sucrose concentration 30 g/L, temperature 26°C, shaking frequency 180 rpm, shaking diameter 5 cm. Experimental cultivation conditions for *E. coli* K12: Flask volume 250 mL, filling volume 5 mL, TB-medium; temperature 37°C, shaking frequency 300 rpm, shaking diameter 5 cm. *E. coli* data were kindly provided by Dr. Matthias Funke. Maximum growth rates (\(\mu_{\text{max}}\)) were determined from the exponential growth phase of the OTR-curve. The OTR-curve was plotted as In OTR over time to apply a linear regression.

The OTR of all three cell lines showed a triangle shape which is typical for tobacco suspensions (Figure 3-2A). The highest value of the OTR was achieved with the BY-2\(_{\text{IME}}\) cell line after 110 h with 8.3 mmol/L/h. The NT-1 cell line obtained the lowest OTR-value (6.4 mmol/L/h) of the three cultures. Maximum growth rates (\(\mu_{\text{max}}\)) were determined from the exponential growth phase of the OTR-curve. The OTR-curve was plotted as In OTR over time to apply a linear regression. The highest maximum growth rate during the exponential growth phase of \(\mu_{\text{max}} = 0.04\) l/h was obtained for the BY-2\(_{\text{IME}}\) cell line. This is comparable to the data of NAGATA ET AL. (1992) who obtained a maximum growth rate of 0.044 l/h for BY-2 suspension cells. These cells are characterized by the highest growth rate for plant suspension cells (TATICEK ET AL. 1991). Under the given culture conditions, a maximum growth rate of
0.038 1/h for BY-2_{DAS} and a maximum growth rate of 0.029 1/h for NT-1 cells was determined (Figure 3-2A).

For classifying the magnitude of a tobacco OTR, it is shown together with an OTR of a non-oxygen limited growth of \textit{E. coli} in Figure 3-2B. Here, the maximum OTR-values as well as the growth rates of both cultures were in totally different magnitudes. The highest OTR-value of \textit{E. coli} was over 15-times and the maximum growth rate ($\mu_{\text{max}}$) was over 20-times higher than that of the BY-2 cell line. Due to this low growth rate and the fact that no selection markers were used in the MS-medium, a high risk of contamination persisted for plant cell culture. In the experiments performed within this thesis, the contamination rate was below 5% (5 of 100 shake flasks were contaminated).

### 3.3 Characterization of \textit{Nicotiana tabacum} cell growth under given cultivation conditions

In the following experiments, BY-2 and NT-1 cell growth was assayed under given IME and DAS cultivation conditions (2.4.2). For this characterization several offline parameter were determined. This chapter focuses only on the influence of different cultivation conditions and not on a detailed interpretation of nutrient depletions which is addressed in the next Chapter (4). In a first set of experiments, the wild type cell lines of BY-2 and NT-1 were cultivated under IME and DAS cultivation conditions (2.4.2) and given media formulations (2.3).
Figure 3-3: Influence of different cultivation conditions on growth of *Nicotiana tabacum* BY-2 and NT-1 wild type cells in MS-medium. Solid line (closed symbols) represents the data obtained with IME cultivation conditions and dashed line (open symbols) the data obtained with cultivation conditions from DAS. (A, B) Oxygen transfer rates (OTR) and maximum oxygen transfer capacity obtained with a 1 M sulfite system (C, D) packed cell volume (PCV) and dry weight (D, E) osmolality and conductivity. Mean values and error bars of osmolality result from three measurements. (G-J) Consumption of the carbon source. Carbon source was determined via HPLC and mean values and error bars result from three HPLC measurements. (K, L) pH and phosphate concentration. Experimental cultivation conditions (IME): Flask volume 250 mL, filling volume 50 mL, temperature 26°C, shaking frequency 180 rpm, shaking diameter 5 cm. Experimental cultivation conditions (DAS): Flask volume 250 mL, filling volume 50 mL, temperature 25°C, shaking frequency 130 rpm, shaking diameter 5 cm.
Figure 3-3 represents the oxygen transfer rate (OTR), the maximum oxygen transfer capacity (OTR\textsubscript{max}) and various offline parameters of BY-2 and NT-1 wild type cells over cultivation time. The black line indicates the maximum oxygen transfer capacity for IME (solid) and DAS (dashed) cultivation conditions (Figure 3-3A and B). For the determination of OTR\textsubscript{max} in shake flasks the sulfite method was used to simulate a biological oxygen consumer (HERMANN 2001). An OTR\textsubscript{max} of 10.6 mmol/L/h for IME and of 6.7 mmol/L/h for DAS cultivation conditions was measured. The higher the shaking frequency the faster is the renewal of the liquid film in the shake flask and, therefore, the specific mass transfer area which is proportional to the OTR\textsubscript{max}. Besides the shaking frequency there are other operating parameters which also influence the OTR\textsubscript{max} in shake flasks (MAIER AND BÜCHS 2001). These operating parameters are:

- flask size and shape
- filling volume
- shaking diameter

When comparing IME and DAS cultivation conditions, the flask size, filling volume and shaking diameter were identical, so that the difference in the OTR\textsubscript{max} could be directly contributed to the different shaking frequency. The influence of the different cultivation temperature of 1°C was neglected. The measured maximum OTR-value of the cells was lower than the preceding OTR\textsubscript{max}, indicating no oxygen limiting conditions. Foremost, the influence of the given cultivation conditions on the growth of BY-2 wild type cells was investigated.

Figure 3-3A shows the OTR profiles of BY-2 wild type cells in MS-medium under IME and DAS operating conditions with different shaking frequencies and temperatures. A higher shaking frequency and a higher temperature resulted in a faster increase of the OTR and an approximately 20% higher OTR (Figure 3-3A). The maximum growth rate of BY-2 cells under IME conditions was 0.042 1/h and under DAS conditions only 0.036 1/h. The PCV had a final value of 75 % for IME cultivation conditions and only 58% for DAS conditions. Under both conditions the highest values were obtained after 168 h of cultivation (Figure 3-3C). As the PCV measurement has a large error in the beginning of the cultivation, it was no longer used within this work. Moreover, a 16% higher dry weight concentration with a maximum value of 14.9 g/L was obtained after 120 h under IME conditions. In the last two days the dry weight decreased to 12.8 g/L for the culture at IME conditions (Figure 3-3C). The values for the osmolality and conductivity remained nearly constant during the first 80 h of cultivation. After 96 h, both values decreased rapidly almost to zero at which the decrease under IME
conditions was temporally earlier (Figure 3-3E). Sucrose was hydrolyzed into its building blocks glucose and fructose which were consumed by the cells. According to Figure 3-3G and I, the carbon consumption under IME conditions was faster, which is in good agreement to the faster cell growth. Figure 3-3K shows the pH-value and the consumption of the phosphate concentration over time (h). No significant variations were observed between both cultivation conditions. After a strong acidification from 5.6 to pH 4.3 within the first 24 h, the pH increased to its initial value during the cultivation. The phosphate concentration was almost constant during the first 48 h of the cultivation. Subsequently, it was consumed by the cells in a period of 48 h under IME and 72 h under DAS conditions, respectively.

The oxygen transfer rates of the NT-1 nearly superimposed from both cultivations (Figure 3-3B). The peak of the OTR obtained with DAS conditions was 8 h later than that obtained with IME conditions. However, the maximum value of the NT-1 cells was still approximately 20% lower compared to that of BY-2 cells. In terms of growth parameters, the NT-1 cell line grew quite similar to the BY-2 cell line. However, the phosphate uptake was different (Figure 3-3K and L). Independent of the cultivation conditions, phosphate was consumed within the first 24 h. Thus, a possible explanation for the lower OTR of the NT-1 cells could be attributed to a phosphate limitation in the MS-medium. This hypothesis is investigated in Chapter 5.3.1.

In conclusion, a higher shaking frequency and a slightly higher cultivation temperature lead to a higher OTR, a faster cell growth and higher biomass concentrations for BY-2 as well as for NT-1 cells. Based on these observations, IME cultivation conditions were used as standard cultivation conditions in further experiments (unless otherwise stated).
Chapter 4  The \( \text{BY-2}_{\text{CsVMV}} \text{GFP-KDEL} \) cell line: Comprehensive analysis of cell growth and product formation in shake flasks

As the optimal cultivation conditions for BY-2 wild type cells were determined in Chapter 3, the following step was to compare these results in terms of growth and nutrient consumption with a transgenic BY-2 cell line. For the transgenic cell line, additionally, the total soluble protein production was assayed using the Bradford assay and the production of GFP was detected via Western blot and fluorescence analysis. For this characterization the chosen transgenic \( \text{BY-2}_{\text{CsVMV}} \text{GFP-KDEL} \) cell line was used (3.1). As mentioned in section 2.2, this cell line expresses an ER-retarded GFP which could be nicely illustrated by confocal laser scanning microscopy as shown in Figure 4-1.

![Figure 4-1: Picture of three day old \( \text{BY-2}_{\text{CsVMV}} \text{GFP-KDEL} \) cells.](image)

4.1 Characterization of BY-2 cell growth in standard MS-medium

To assay and characterize, in particular, \( \text{N. tabacum} \) BY-2 cell growth, BY-2 cells were cultivated in shake flasks at 26°C for 10 days. All measurements shown in Figure 4-2 were performed in biological triplicates and are represented by mean values.
Cultivation of transgenic *Nicotiana tabacum* BY-2<sub>CaMV</sub>GFP-KDEL cells in MS-medium. (A) Oxygen transfer rate (OTR) (B) wet and dry weight (C) wet/dry weight ratio, osmolality and conductivity (D) pH, nitrate and ammonium concentration (E) phosphate, potassium and sulfate concentration and the consumption of the C-source (F). Experimental cultivation conditions: Flask volume 250 mL, filling volume 50 mL, temperature 26°C, shaking frequency 180 rpm, shaking diameter 5 cm. Mean values and error bars result from three biological triplicates. Black arrow indicates a change in the breathing activity.

Figure 4-2A represents the OTR as a function of time (h). The OTR-curve was characterized by a monotonous increase. After 94 h (indicated by the black arrow), an interruption of the breathing activity by a reproducible characteristic small peak and a shift to a different breathing activity was detected. After 120 h, the OTR reached a maximum which coincided with the depletion of the carbon sources (Figure 4-2F). Figure 4-2B shows the biomass concentration represented by the wet and dry weight. Until 144 h, both curves increased in
parallel up to values of 330 g/L for the wet weight and a maximum concentration of 14 g/L for the dry weight. After 144 h, the wet weight increased to its maximum value over 400 g/L whereas the dry weight started to decrease. The maximum of dry weight concentration corresponded approximately to the maximum value of the OTR and the depletion of the carbon source. Once the carbon source was depleted, no more cell growth was observed. The culture broth was similar to “distilled water” with a low conductivity and osmolality (Figure 4-2C). As a consequence the wet/dry weight ratio increased and water flushed into the cells due to the higher osmotic pressure in the cell (Figure 4-2C). This increase of the wet/dry weight ratio influenced also the cell morphology as it could be illustrated by microscopic pictures of this cell line.

Figure 4-3: BY-2<sub>CaMV</sub>GFP-KDEL cells cultivated in MS-medium in different stages of the cultivation. Experimental cultivation conditions: Flask volume 250 mL, filling volume 50 mL, temperature 26°C, shaking frequency 180 rpm, shaking diameter 5 cm (Light microscopy, 400x magnification).

Figure 4-3 depicts typical morphological changes of BY-2 cells during the course of cultivation. Directly, after subcultivation, BY-2 cells showed a round shape and large vacuoles (Figure 4-3A). In the exponential phase, cells re-synthesized cell organelles and a dense, shrunk cytoplasm was visible (Figure 4-3B). As osmolality decreased by nutrient consumption, the cell volume increased (Figure 4-3C) and often single cells larger than 100 µm were formed. Here, cells were characterized by a round shape due to an expanded vacuole (Figure 4-3D). This process is called deplasmolysis (1.2.2). In addition to transmission light pictures, the deplasmolysis of BY-2 cells could also be clearly illustrated by fluorescence pictures of the cells as shown in Figure 4-4.
According to Figure 4-4, the expressed GFP accumulated in the ER around the nucleus (Figure 4-4A) and the cytoplasm (Figure 4-4B and C) (indicated by the red arrows). The black area in the cell is the vacuole where no GFP is accumulated. Occasionally, single cells of over 120 μm were found (Figure 4-4D).

The values for the osmolality and conductivity remained nearly constant in the first 80 h of the cultivation (Figure 4-2C). Since the two monomers, glucose and fructose, resulting from sucrose hydrolysis lead to a higher osmotic pressure than non-hydrolyzed sucrose, the osmolality remained nearly constant although sugars were consumed. Between 96 h and 120 h, both values decreased almost to zero at the end of the cultivation. This indicated that after 168 h, roughly all nutrients in the medium were depleted and further cell growth and protein production was no longer possible. As sugar is responsible for half of the osmolality, the decrease in the osmolality followed the decrease of the carbon source. Conductivity measurements have also been used to monitor cell growth by (HAHLBROCK AND KUHLEN 1972). A linear relationship between cell growth of BY-2 cells and a decrease in conductivity was observed. One disadvantage of this approach is that this method is indirect and, therefore, the decrease in the dry weight at the end of batch fermentations was not monitored. This might result from the fact that cell lysis at the end of a fermentation is not monitored by conductivity measurements (KWOK ET AL. 1992).

Figure 4-2D shows the pH-value and the consumption of the two nitrogen sources in the MS-medium ammonium and nitrate. With a concentration of 60.02 mM nitrogen is the major constituent in the MS-medium. As shown by Figure 4-2D, ammonium was consumed first, whereas the nitrate concentration remained constant within the first 48 h. Afterwards, both
The BY-2\textsubscript{C\textsc{a}V\textsc{m}yGFP-KDEL} cell line:
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Nitrogen sources were consumed in parallel whereas ammonium was depleted first (96 h). Nitrate was no longer detectable after about 140 h. Further nitrogen sources for tobacco cells are urea and amino acids. Urea can be used as a nitrogen source, but the growth is slower than that in a mixture of ammonium and nitrate (Kirby\ et\ al.\ 1987). Amino acids (in a defined mixture) can also be used as a sole nitrogen source for the growth of tobacco cells (Müller\ and\ Grafe\ 1978), but since amino acids are more expensive than ammonium, nitrate and urea a use for culture purposes is unprofitable. During batch cultivations, the culture showed a typical pH trend for BY-2 suspension cultures (Figure 4-2D) (Holland\ et\ al.\ 2010). The preferential uptake of ammonium resulted in a decrease in pH-values in the first 24 h of the cultivation. After 48 h, nitrate utilization was increased and a simultaneous increase of pH during the last stage of the cultivation occurred. This pH trend has also been shown for Ipomoea sp. cultures (Martin\ et\ al.\ 1977).

Figure 4-2E depicts the consumption of three macronutrients: phosphate, potassium and sulfate. In biological systems, phosphate plays an important role in energy transfer via the formation of the pyrophosphate bond in ATP. Moreover, phosphate is an essential component of macromolecules such as nucleic acids and phosphor lipids. The consumption of phosphate by BY-2 cells from the culture medium is exceptionally rapid compared to other major inorganic constituents, such as nitrate and potassium (Kato\ and\ Nagai\ 1979). According to Figure 4-2E the initial phosphate concentration in the MS-medium is 2.7 mM, and after approximately 84 h no more phosphate was detected in the supernatant. This depletion of phosphate was considered to be bound to myo-inositol to form phytic acid, which is the principal storage form of phosphor in plants (Mitsuhashi\ et\ al.\ 2005). In plants, phosphate is released by the enzyme phytase (Brinch-Pedersen\ et\ al.\ 2002). As phosphate was bound in phytic acid, it was no longer detectable in the culture broth with the applied test kit (2.6.5). Potassium is the major cation within the MS-medium and hence, contributes significantly to the osmotic potential of plant cells. Within the first 100 h of cultivation, only 6 mmol/L potassium was consumed by the cells. Thereafter, a rapid consumption of potassium took place and after 144 h the potassium source was depleted. A potassium deficiency can lead to hyperhydricity (Pasqualetto\ et\ al.\ 1988) and results in a decrease of phosphate absorption (Chin\ and\ Miller\ 1982). Sulfur is mainly absorbed as \text{SO}_4^{2-}, which is the usual source in plant culture media. As seen in Figure 4-2E sulfate was constantly consumed and after 144 h no more sulfate was detectable. Since sulfur is responsible for the formation of S – S bridges, the protein synthesis as well as the growth of tobacco suspension cells were reduced when...
cultivated in a medium containing only 0.6 mM SO\textsubscript{4}\textsuperscript{2-} instead of 1.7 mM (KLAPHECK ET AL. 1982).

As only a limited number of autotrophic plant cell lines have been isolated for in vitro cultivation, most plant suspension cells inclusive tobacco, are heterotrophic and, thus, a carbon source has to be incorporated in the medium. For tobacco suspension cultures in shake flasks, 30 g/L sucrose is favorable, as the highest cell concentrations could be attained with this carbon source concentration (MUSHARIGE AND SKOOG 1962). Figure 4-2F distinctly shows the sucrose depletion resulting from the hydrolysis of sucrose to its monomers glucose and fructose followed by their sequential consumption, with glucose being preferentially utilized. The hydrolysis of sucrose is catalyzed by extracellular invertases (KING AND STREET 1977) or by invertases located in the cell wall (YOSHIDA ET AL. 1973). A direct comparison of the OTR and the carbon source illustrated that once carbon was depleted (after about 122 h), the OTR started to decrease.

The yield coefficient $Y_{XS}$ – the ratio of produced biomass to the amount of the utilized substrate – determined to be 0.46 g/g ± 0.05 which agrees with the findings of other authors (KATO AND NAGAI 1979; MUSHARIGE AND SKOOG 1962) for BY-2 cells. Using 30 g/L glucose instead of 30 g/L sucrose resulted in a slower cell growth (data not shown). However, there are plant strains such as the alder tree species (*Alnus crispa* and *Alnus cordata*) that prefer glucose as a nutrient and show faster cell growth with glucose (TREMBLAY AND LALONDE 1984). KRONER AND KUKULCZANKA (1985) discovered that tips of the flower *Canna indica* survived better in a mixture of 25 g/L glucose and 5 g/L sucrose instead of 30 g/L sucrose.

With respect to the growth behavior and nutrient consumption, no significant differences between the wild type and the transgenic cell line were observed. Similar maximum OTR-values as well as similar yield coefficients have been achieved although the inoculation density of the transgenic cell line was 5% (wild type 2%) (Figure 3-3 and Figure 4-2). Thus, the constitutive expression of the recombinant protein (GFP) resulted in an additional metabolic burden during plant cell growth and hence, reduced the specific growth rate. To compensate that additional metabolic burden, transgenic cell lines were inoculated with a higher cell density. The analysis of the recombinant protein production is addressed in the following Chapter.
4.2 Analysis of the total soluble protein concentration and GFP formation

This analysis was performed in parallel to the aforementioned experiment (4.1; Figure 4-2). Here, the formation of the total soluble protein and the recombinant protein GFP was measured for the BY-2CsVMVGFP-KDEL cell line. The green fluorescent protein (GFP) is one of the most frequently employed molecular reporters for gene expression in living cells. Its use in monitoring gene expression and protein localization has been well documented (CamPELL and ChoY 2001; Tsien 1998). Via the KDEL sequence the expressed GFP is retarded at the endoplasmatic reticulum (Munro and Pelham 1987). Figure 4-5A and B represent the protein concentration and Figure 4-5C the GFP formation visualized by Western blot.
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Figure 4-5: Analysis of the protein and GFP formation via SDS-PAGE and Western blot over time. (A) Protein concentration plotted over time (B) SDS-PAGE (C) Western blot.

Experimental cultivation conditions: Flask volume 250 mL, filling volume 50 mL, temperature 26°C, shaking frequency 180 rpm, shaking diameter 5 cm. Mean values and error bars result from three biological triplicates. For the SDS-PAGE 10 µL supernatant of 0.1 g disintegrated wet weight was separated on a NuPage® 4-12% (w/v) Bis-Tris gel and stained with Coomassie brilliant blue. For the Western blot the gel was transferred to a nitrocellulose membrane. Target protein bands were specifically detected with Rabbit-α-TurboGFP and Goat-α-Rabbit IgG<sub>AP</sub> and were finally visualized by the NBT-BCIP reaction. M: 3 µL protein marker (Page Ruler). 0-240 h: every day samples of cells. 12.5 (+) and 25 ng (++) pure TurboGFP as a positive control.

The protein concentration determined by a Bradford assay is displayed with respect to two different dimensions (Figure 4-5A): On the one hand related to a specific amount of cell mass (0.1 g); and on the other hand related to the total amount of soluble protein. In the latter case, the specific amount is multiplied by the wet weight of the culture, representing the total
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amount of soluble protein. By doing so, osmotic influences were considered. The wet weight related protein concentration (closed symbols) increased during the first 72 h, to a maximum protein concentration of 7 mg/gFW. After 72 h it declined severely to concentrations lower than 1 mg/gFW and remained at this level until the end of the cultivation. The band intensities of the SDS-PAGE were in correspondence with the determined protein concentrations (Figure 4-5B). The total soluble protein concentration increased to a maximum value of 700 mg/L after 96 h of cultivation (Figure 4-5A). Although the wet weight increased until the end of the cultivation (Figure 4-2B), the protein decreased to a third of its maximum value within 24 h and remained at these low concentrations (Figure 4-5A).

In Figure 4-5C the respective Western blot of the SDS-PAGE is illustrated. GFP is a dimer and has a size of 27 kDa (TsiEN 1998). The red box indicates the position of the expressed GFP on the gel (Figure 4-5B) and the blot (Figure 4-5C). GFP and even the band intensities of the positive control were not detectable on the gel since the expression level as well as the concentration of pure TurboGFP was too low. A detection limit of approximately 100 ng was reported for gels stained with Coomassie blue (Shevchenko et al. 1996). On the Western blot, however, bands of the expressed GFP and the positive control were visible. By means of the Western blot (NBT/BCIP staining), proteins could be detected down to limits of about 5 ng/L (La Clair et al. 2004). In addition to the band at 27 kDa, the positive control showed a minor band at about 56 kDa which could be agglomerated GFP. Moreover, the band of the positive control laid just under the GFP-band of the samples which could be attributed to the KDEL sequence attached at the GFP. The reason for the second minor band at about 24 kDa was not clear. It can be seen, that the band intensity increased within the first 72 h. After 96 h, the band intensities decreased dramatically and were barely visible (Figure 4-5B and C).

One explanation for this decrease could be a dilution effect caused by osmosis. After 120 h, the osmolality of the medium was extremely low (0.036 Osm/kg) (Figure 4-2C). Through deplasmolysis, water is flushing into the cells, as the osmotic pressure in the cell is higher than in the medium (Figure 1-2). Thus, in 0.1 g wet weight – which was used for the Bradford assay – the water content of the cell increased with the cultivation time. Further explanations for the decrease of the protein content in the cells are either enhanced protein secretion in the stationary phase (Winicur et al. 1998) or a proteolytic degradation induced by nutrient starvation (Moriyasu and Ohsumi 1996). This topic is extensively discussed in Chapter 9.3.

In addition to the Western blot, GFP was also determined by the measurement of the fluorescence (Figure 4-6). Similar to the protein concentration (Figure 4-5A), also the
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Fluorescence of GFP can be measured related either to a certain mass (closed symbols) or a certain volume of cells (open symbols). As a negative control the fluorescence of the wild type was measured under identical conditions. The results are also shown in Figure 4-6.

**Figure 4-6:** Fluorescence intensity of BY-2CsVMV-GFP-KDEL and wild type cells determined offline in a MTP. Experimental cultivation conditions: Flask volume 250 mL, filling volume 50 mL, temperature 26°C, shaking frequency 180 rpm, shaking diameter 5 cm. Mean values and error bars result from three biological triplicates. Measurement of the fluorescence: 48-well MTP, Ex. 485 nm, Em. 520 nm; gain 30. Wet weight related (closed symbols): 0.1 g wet weight resuspended in 500 µL supernatant; volumetric (open symbols): 500 µL of culture broth.

The BY-2 wild type showed a constant specific fluorescence intensity level of about 800 – 1000 RFU. Since the pure MS-medium had lower fluorescence values of about 450 – 500 RFU (data not shown), the higher fluorescence of the BY-2 wild type could be attributed to the auto fluorescence of various cell internal fluorophores (Koenig and Schneckenburger 1994; Ramanujam 2002).

The specific fluorescence (closed symbols) of the transgenic BY-2 cells followed the trend of the Western blot (Figure 4-5C). It increased to its maximum value of 8500 RFU after 72 h, followed by a sharp decrease to a minimum value of 2200 RFU after 120 h which was barely higher compared to the auto fluorescence of the wild type. Surprisingly, the fluorescence increased again to a value of 8000 RFU within just 24 h while simultaneously no band was detectable on the Western blot (Figure 4-5C). The volumetric fluorescence (open symbols) increased almost linear and reached its maximum value after 96 h of cultivation. Afterwards the signal decreased by approximately 25% and remained nearly constant at this value until the end of the cultivation (Figure 4-6).

In this experiment two totally different methods for the detection of the target protein (GFP) were applied. In the BY-2CsVMV-GFP-KDEL cell line GFP is ER-retarded and therefore, cells have to be disintegrated for the determination of GFP via Western blot. The GFP fluorescence signal, however, is a composite signal that can be fatally affected by the concentration of
living cells and their metabolism (STÄRK ET AL. 2002) and the inner filter effect (IFE) as well as fluorescence quenching (SU ET AL. 2005). Especially the IFE may have a tremendous influence on the GFP fluorescence signal, as plant cells tend to form aggregates and reach high biomass concentrations. Furthermore a danger of interference with auto fluorescence cannot be excluded. Thus, an accurate estimation of GFP from culture fluorescence is susceptible for misinterpretation. Hence, the Western blot instead of the fluorescence signal was used in further experiments for the quantification of the GFP amount produced by the cells.

4.3 Detection of nutrient depletions on the basis of the OTR

Based on the results discussed in section 4.1, it was not yet clarified what happened at the assumed transition in the breathing activity, which is indicated in the OTR-curve by a black arrow (Figure 4-2A). It either indicated a complete consumption of sucrose, phosphate or that of ammonium since these three concentrations were depleted almost in the same time period. In order to improve the understanding of this phenomenon and its resulting impact on cell metabolism, an additional experiment with increased sample number was conducted. To assure comparability, the cultivation and sample analysis was performed under exactly the same conditions as in the previous experiment. Moreover, the number of the offline samples was increased from four to eighteen samples in the time period between 48 h and 120 h.
The BY-2<sub>CsVMV</sub>GFP-KDEL cell line:
Comprehensive analysis of cell growth and product formation in shake flasks

Figure 4-7A shows the OTR, dry weight and the wet/dry weight ratio as a function of time. The solid line represents the highest value of the OTR after 108 h. The curve of the OTR and the dry weight showed exponential growth and were nearly superimposed until 106 h which suggests a good correlation between the OTR and the dry weight. The highest dry weight concentration (16.9 g/L) was reached after 111 h which correlated to the highest value of the OTR with 6.2 mmol/L/h after 105 h.

After reaching the highest value of the OTR the dry weight decreased to 13.9 g/L. The wet/dry weight ratio, however, nearly doubled in the same time indicating that due to osmosis only water is flushing into the cells. Figure 4-7B distinctly shows the sucrose depletion (dotted line) resulting from the hydrolysis of sucrose to its building blocks glucose and fructose followed by their consumption. The highest value of the OTR (represented by the solid line) corresponded with the depletion of the carbon source. Figure 4-7C represents the OTR-curve and the consumption of the two nitrogen sources, ammonium and nitrate over time. Here, the shift in metabolism and, therefore, in the OTR (after 88 h) correlated well with the depletion of the ammonium concentration in the supernatant (marked by the dashed line).

Hence, for the first time the depletion of ammonium in MS-medium could be correlated to an online signal of the OTR.
Under the applied cultivation conditions the OTR can be used as an online signal for the depletion of the carbon source, for the depletion of ammonium and for the time point where the highest dry weight concentration is achieved and is hence, an important tool for further media optimization. For the transgenic NT-1CsVMVHA cell line the same characterization was performed and the results of this experiment are presented in 5.3.1. The RAMOS device has been employed in other projects with various organisms to detect nutrient limitations and to optimize medium ingredients, i.e. A. indica (Raval et al. 2003), E. coli (Losen et al. 2004), H. polymorpha (Kottmeier et al. 2009) and S. pombe (Klement et al. 2011).

In summary, this investigation has shown for the first time a comprehensive characterization of a transgenic Nicotiana tabacum BY-2 cell line by combining online and offline analysis of multiple parameters. The RAMOS device, which allows the online analysis of oxygen consumption has been proven to be a useful analytical tool, as changes of cell metabolism could be easily detected online.
Chapter 5  Media optimization

It has already been demonstrated that recombinant protein production can be further improved by manipulating mineral media compositions (ZHONG 2001; HOLLAND ET AL. 2010). This chapter deals with the optimization of the MS-medium based on the standard MS-medium published by MUSHARIGE AND SKOOG (1962). In a set of different experiments, the concentrations of single nutrients were changed in order to analyze the influence of these individual nutrients on plant cell metabolism and recombinant protein production. In a first experiment performed in the RAMOS device, plant cell growth in the commercial MS-medium was compared with plant cell growth in the MS-medium, which was produced from self-made stock solutions. Thereby, it should be investigated if plant cell growth is equal for both media. The determined oxygen transfer rates did not deviate by more than 5% among each other (data not shown). Thus, it was proven that the commercial MS-medium can be reproduced from self-made stock solutions. As nitrogen plays a pivotal role in protein synthesis (PADGETT AND LEONARD 1993), the media optimization is focused on the effects of different ratios and different initial amounts of the two nitrogen sources nitrate and ammonium.

5.1 Influence of the nitrate to ammonium ratio on BY-2 cell growth

The two nitrogen sources in the MS-medium are ammonium (20.6 mM) and nitrate (39.4 mM) (MUSHARIGE AND SKOOG 1962). From the literature it is known that the ratio of the two nitrogen sources has significant influence on plant cell metabolism (BELLINI ET AL. 1990; SELBY AND HARVEY 1990). In contrast to nitrate the depletion of ammonium causes a reproducible change in the breathing activity (Figure 4-2A). Therefore, the following experiment addresses how, in particular, the initial nitrate to ammonium ratio effects plant cell metabolism. Figure 5-1 represents the oxygen transfer rates of BY-2CsVMVGFP-KDEL cells in modified MS-medium containing different nitrate to ammonium ratios as a function of time.
Figure 5-1: Cultivation of transgenic *Nicotiana tabacum* BY-2 cells in modified MS-medium with decreasing initial ammonium concentrations. Oxygen transfer rates (OTR) of BY-2 cells growing in standard MS-medium containing 20.6 mM NH$_4^+$ and 39.4 mM NO$_3^-$ (black) and in modified MS-medium containing 10 mM NH$_4^+$ and 50 mM KNO$_3$ (gray line) or 60 mM KNO$_3$ (light gray). Experimental cultivation conditions: Flask volume 250 mL, filling volume 50 mL, modified MS-medium (2.3.2), temperature 26°C, shaking frequency 180 rpm, shaking diameter 5 cm. Dashed lines represent the assumed depletion of ammonium.

A graduated growth pattern resulted in dependence of the initial ammonium concentration from this experiment (Figure 5-1). The reference culture (black line) contained 20.61 mM ammonium and 39.4 mM nitrate (simplified to 20:40) and showed the expected pattern for BY-2 cells under these cultivation conditions. A shift from ammonium to nitrate at a constant nitrogen concentration of 60 mM resulted in slower cell growth (Figure 5-1). The growth rate as well as the highest OTR-value decreased with a decreased initial ammonium concentration. Consequently, a mixture of both nitrate and ammonium resulted in a better vegetative growth and hence, in higher OTR-values than supplying nitrate alone. In a medium containing ammonium as a sole nitrogen source, no BY-2 cell growth was detected (data not shown). However, BY-2 cells can grow in a medium with ammonium as the sole nitrogen source if Krebs cycle acids such as citrate, malate, fumarate or succinate are added to the MS-medium (GAMBORG 1970). In the modified MS-medium containing 50 mM nitrate (gray) or containing nitrate as the sole nitrogen source (light gray) a significantly slower growth compared to that of the reference culture was observed. More ammonium gives the cell an advantage as the energy-consuming reduction of nitrate is avoided (Figure 1-1).

Conclusively it was demonstrated that supplying a mixture of both nitrate and ammonium resulted in better vegetative growth and enhanced nutrient accumulation than either form alone. These findings concur well with those of other studies (HAGEMAN ET AL. 1962; HAYNES AND GOH 1978). It was clearly shown that a higher ammonium to nitrate ratio (at a total nitrogen concentration of 60 mM) had a beneficial effect on plant cell growth (Figure 5-1). Furthermore it is known that ammonium plays a key role in protein synthesis (GAMBORG
1970). Based on the previously discussed results the next step was to investigate if an increase in the initial amount of ammonium would have a beneficial effect on plant cell growth and protein production. This hypothesis has been investigated in the following experiments.

5.2 Influence of an increased ammonium concentration on the metabolism of BY-2 and NT-1 cells

Considering that a mixture of ammonium and nitrate in the MS-medium is essential for optimal cell growth and considering that the initial osmotic pressure plays an essential role in plant cell metabolism, the intention was to increase the initial amount of ammonium in the MS-medium without increasing the initial osmotic pressure. Moreover, both nitrogen sources have to be supplied. To achieve this, the ammonium concentration was increased in the same concentration as that of potassium was decreased. The concentration differences of the relevant nutrients are shown in Figure 5-2. In the modified MS-medium the total amount of nitrogen was 10 mM higher than in the standard MS-medium. However, the initial amount of potassium was simultaneously decreased to 10.05 mM.

![Figure 5-2: Concentrations of ammonium, potassium and nitrogen of the modified MS-medium. Units of the initial concentrations of ammonium, potassium and nitrate are given in mM.](image)

Figure 5-3 depicts the data obtained with the standard MS-medium (solid symbols) and with the modified MS-medium (open symbols). The initial osmotic pressure in the modified (ammonium enriched) MS-medium was equal to the osmotic pressure in the standard MS-medium.
Figure 5-3: Cultivation of transgenic *Nicotiana tabacum* BY-2_CMVnGFP-KDEL cells in MS-medium (solid line) and modified MS-medium (dashed line). (A) Oxygen transfer rate (OTR) and ammonium concentration, (B) wet and dry weight, (C) GFP and protein concentration related to cell broth. Experimental cultivation conditions: Flask volume 250 mL, filling volume 50 mL, modified MS-medium (2.3.2), temperature 26°C, shaking frequency 180 rpm, shaking diameter 5 cm. Dotted lines represent the expected exhaustion of the ammonium concentration indicated by the bend in the OTR-curve.

Figure 5-3A shows the impact of the higher initial amount of ammonium on the OTR. Despite the same initial biomass concentration, a different growth behavior was observed compared to the standard MS-medium. The growth in the modified MS-medium was faster ($\mu_{\text{max}} = 0.036 \, \text{1/h}$ in the modified MS-medium and 0.034 \, 1/h in the standard MS-medium) and the highest OTR-value was about 15% higher. The characteristic shift in metabolism was shifted to a later time (from 95 h in the standard MS-medium to 100 h in the modified MS-
Media optimization

medium). The shift correlated well with the exhaustion of the ammonium ions in the respective MS-medium. As seen in Figure 1-1, ammonium is a readily metabolizable source of nitrogen and can be incorporated directly into the nitrogen pathway. An increase of the initial nitrate concentration, however, resulted in a significantly slower cell growth (Figure 5-1). Thus, it is more advantageous for the BY-2 cells from an energy standpoint to use ammonium instead of nitrate because the energy consuming reducing step from nitrate to ammonium is avoided. Consuming ammonium directly resulted in faster growth and a higher biomass concentration (Figure 5-3B). The total protein concentrations as well as the GFP concentration were significantly higher in the ammonium-enriched MS-medium compared to those in the standard MS-medium (Figure 5-3C). After 144 h, the maximum GFP concentration nearly doubled (1.9-fold increase). Further offline parameters (conductivity, osmolality, pH-value, carbon source) were also analyzed in the standard MS-medium as well as in the ammonium-enriched MS-medium. Between both media, no significant variations of the respective values were observed (data not shown).

In a further experiment, it was investigated if the increased ammonium concentration would also affect another transgenic tobacco cell line and boost the production of a different recombinant protein. For this purpose the transgenic N. tabacum cell line NT-1, which is closely related to the BY-2 cell line (NAGATA AND KUMAGAI 1999), was used. It produces the pharmaceutically relevant protein influenza hemagglutinin (HA), which is targeted to the endomembrane system of the plant cell.
Figure 5-4: Cultivation of transgenic HA producing *Nicotiana tabacum* NT-1 cells in MS-medium (solid line) and modified MS-medium (dashed line). (A) Oxygen transfer rate (OTR) and ammonium concentration, (B) wet and dry weight, (C) HA and protein concentration related to cell broth. Experimental cultivation conditions: Flask volume 250 mL, filling volume 50 mL, modified MS-medium (2.3.3) temperature 26°C, shaking frequency 180 rpm, shaking diameter 5 cm. Dotted lines represent the expected exhaustion of the ammonium concentration indicated by the bend in the OTR-curve.

Figure 5-4 represents the data obtained with standard MS-medium (solid symbols) and with modified (ammonium-enriched) MS-medium (open symbols) for the transgenic *N. tabacum* NT-1_CaVMVHA cell line. The experimental set-up was identical to that in the experiment illustrated in Figure 5-3. Figure 5-4A shows the OTR-curves and the ammonium concentration of the NT-1 cell culture supernatant in the standard and the ammonium-enriched MS-medium. In the first 82 h of the cultivation, the growth pattern in both MS-
media was identical. Once ammonium was depleted in the standard MS-medium, the OTR of the NT-1 cells declined. As a result the fresh weight of the cells cultivated in the standard MS-medium was lower (Figure 5-4B). Figure 5-4C depicts the total protein concentration together with the concentration of the influenza hemagglutinin (HA) protein. The pattern of both protein curves was similar. Both curves increased within the first 96 h of the cultivation whereas the protein concentration in the ammonium-enriched medium was approximately 20% higher compared to that in the reference medium. During the last 48 h of the cultivation, the concentration of proteins decreased. As previously demonstrated for the BY-2 cell line (Figure 5-3C), the positive effect of the ammonium-enriched medium on the target protein formation was also confirmed for the NT-1 cell line. After 120 h, the HA concentration also doubled in the modified MS-medium.

5.3 Further media optimization with the NT-1 cell line

In Chapter 5.2 it was demonstrated that an increase in the initial ammonium concentration resulted in a faster cell growth and higher recombinant protein concentration. This increase in ammonium simultaneously changed the ratio of nitrate ions to ammonium ions (Figure 5-2). In this chapter, a systematic approach of MS-media optimization for the transgenic NT-1 CsVMV HA cell line is presented. Three different subjects have been examined:

1) Influence of the initial phosphate concentration on NT-1 cell growth.

2) Influence of three different nitrate/ammonium ratios at a constant nitrogen concentration of 60 mM on NT-1 cell growth.

3) Influence of an increased initial nitrogen concentration of 80 mM on NT-1 cell growth.

How the increased nitrogen concentration affects protein synthesis and recombinant protein production is addressed in the next chapter. For simplification purposes the legend, “20:40” means that this medium contains 20.6 mM ammonium and 39.4 mM nitrate. This nomenclature is used for the different ammonium and nitrate concentrations throughout the complete chapter.

5.3.1 Influence of the initial phosphate concentration and nitrate/ammonium ratio at an initial nitrogen concentration of 60 mM

The initial phosphate concentration of the MS-medium for NT-1 cells is approximately 25% lower compared to that of BY-2 cells (2.3). The aim of this experiment was to examine the influence of three different initial phosphate concentrations and the effect of three different
nitrate/ammonium ratios on NT-1\textsubscript{CVMVHA} cell growth. The three different initial ammonium and nitrate concentrations and their respective ratio are listed in Table 5-1.

<table>
<thead>
<tr>
<th>Ammonium concentration [mM]</th>
<th>Nitrate concentration [mM]</th>
<th>Nitrate/ammonium ratio [-]</th>
</tr>
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<tbody>
<tr>
<td>10</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>20.6</td>
<td>39.4</td>
<td>1.91</td>
</tr>
<tr>
<td>30</td>
<td>30</td>
<td>1</td>
</tr>
</tbody>
</table>

The respective results are presented in Figure 5-5.

The initial phosphate content of the MS-medium for NT-1 cells was 2.04 mM with an ammonium concentration of 20.61 mM and a nitrate concentration of 39.4 mM. This culture did not exhibit the typical triangle shape as shown previously for BY-2 cells (Figure 4-2A).
Derived from these results, cultivations with 1.25 mM and 4.08 mM phosphate were performed to represent a low and a high phosphate concentration, respectively. The culture containing 4.08 mM phosphate and an ammonium nitrate ratio of 20.61 mM to 39.4 mM (Figure 5-5C) showed the expected OTR pattern as shown already in Figure 4-2A for BY-2 cells. Phosphate became limiting with initial concentrations of 2.04 mM and 1.25 mM, respectively. The OTR of the limited culture with 2.04 mM stagnated at an OTR of 5 mmol/L/h after 85 h and the culture contained 1.25 mM phosphate showed a plateau with a highest OTR-value of only 2.5 mmol/L/h after 70 h (Figure 5-5A and B). The above presented results clearly showed that after a limitation of phosphate, the cells stagnated in growth and exhibited a prolonged respiration activity due to the on-going consumption of the carbon source for maintenance.

According to Figure 5-5B, with an initial phosphate concentration of 2.04 mM, the nitrate/ammonium ratio affected the OTR, too. The OTR of the 20:40 and 30:30 experiments with an initial phosphate concentration of 2.04 mM increased to 5.5 mmol/L/h after 122 h whereupon the 10:50 cultivation stagnated at an OTR of 3.5 mmol/L/h after 69 h suggesting an ammonium limitation indicated by the dotted line (Figure 5-5B). In Figure 5-5A both cultivations became phosphate limited after 72 h and therefore, both OTR-curves were superimposed. The influence of different nitrate/ammonium ratios was most apparent at an initial phosphate concentration of 4.08 mM. The respective ammonium depletions are indicated with three dotted lines (Figure 5-5C). Increasing phosphate to 4.08 mM with a simultaneous nitrate to ammonium ratio of 30:30 resulted in the highest OTR of 8.5 mmol/L/h after 96 h. A further increase of the initial phosphate concentration up to 10 mM resulted in the same OTR and in the same biomass concentration compared to 4.08 mM (data not shown).

The initial phosphate concentration had also a strong impact on the biomass concentration represented by the dry weight (Figure 5-5D-F). With increasing phosphate concentration, the maximum dry weight increased from 8.2 g/L at 1.25 mM to 14 g/L at 2.04 mM to the highest dry weight concentration of 16.1 g/L at 4.08 mM phosphate. Besides phosphate, a minimum concentration of 30 mM ammonium was required for an unlimited cell growth. With an initial ammonium concentration of 10 mM, dry weight concentrations of only 10.5 g/L were obtained (Figure 5-5E and F).

The pH showed the expected trend explained already in section 4.1. The earlier ammonium was depleted, the earlier the pH increased due to the consumption of nitrate (Figure 5-5I).
This observation agrees with those of other studies (BEHRENDB AND MATELES 1975; HYNDMAN ET AL. 1981).

It was clearly evidenced that the ratio of nitrate to ammonium ions had a significant effect on the metabolism of plant cells. Even at adequate initial phosphate concentrations NT-1 cells became ammonium limited when supplying just an initial concentration of 10 mM ammonium (Figure 5-5C and F). The effect of an increased initial nitrogen concentration on cell growth and recombinant protein production is addressed in the next chapter.

5.3.2 Influence of an increased nitrogen concentration on NT-1 cell growth and recombinant protein production

To assay and examine in particular the influence of an increased amount of nitrogen at different nitrate to ammonium ratios, NT-1CsVMV-HA were cultivated in modified MS-medium for one week under IME conditions. NT-1 cell growth and recombinant protein production in a modified MS-medium with 80 mM at two different nitrate/ammonium ratios was analyzed and compared to a reference cultivation containing 60 mM nitrogen.
Figure 5-6: Influence of the total nitrogen concentration on NT-1<sub>CAVM</sub>HA cell growth and carbon consumption in modified MS-medium. (A) Oxygen transfer rate (OTR), (B) dry weight, (C) conductivity, (D) pH, (E) sucrose concentration, (F) glucose concentration, (G) fructose concentration and (H) total sugar concentration. Experimental cultivation conditions: Flask volume 250 mL, filling volume 50 mL, modified MS-medium (2.3.3), temperature 26°C, shaking frequency 180 rpm, shaking diameter 5 cm.
Figure 5-6 shows the OTR profiles of NT-1 cells in modified MS-medium by varying the total nitrogen concentration. In the first 82 h of the cultivation, the growth pattern in both modified MS-media was identical. Once ammonium was totally consumed in the 20:40 cultivation, the OTR of the NT-1 cells declined. The highest OTR in this experiment was 6.5 mmol/L/h after 102 h (black squares, reference cultivation). Both other OTR-curves increased with an increasing total nitrogen concentration. Depending on the nitrate to ammonium ratio in both 80 mM cultivations, two different OTR maxima were detected. In the 30:50-cultivation, the highest OTR-value was 9.1 mmol/L/h and in the 40:40-cultivation it was 10 mmol/L/h. This value was simultaneously the highest OTR-value of plant suspension cells detected with the RAMOS device. These results clearly illustrated again, that a lower nitrate to ammonium ratio enhanced plant cell growth since the energy-consuming step of the nitrate reduction is avoided (Figure 1-1). These findings implied a direct connection between the total amount of nitrogen and the highest OTR-value. In all cultivations, sucrose was hydrolyzed into its monomers within the first 80 h of the cultivation (Figure 5-6E). Afterwards both monomers were consumed by the cells at which glucose consumption started earlier (Figure 5-6F and G). In both experiments where 80 mM nitrogen was supplied no more carbon was measured after 120 h of cultivation. Thus, it was not clear whether a depletion of ammonium or the carbon source caused the decrease of the OTR. To proof the major influencing limitation in further experiments more offline samples between 96 and 120 h have to be taken.

Further offline parameters, i.e. dry weight, conductivity and pH (Figure 5-6D), were also analyzed in all different cultivations. Between the different cultivations, no significant variations of the respective values were observed.

How protein synthesis as well as the recombinant protein production of NT-1 cells is affected by cultivation in modified MS-medium with an increased total nitrogen concentration is shown in Figure 5-7. The total soluble protein was measured via Bradford assay and the HA concentration was analyzed by enzyme-linked immunosorbent assay (ELISA).
Figure 5-7: Influence of the total nitrogen concentration on the protein and HA concentration of NT-1<sub>CsvMV</sub>HA cells in modified MS-medium. (A) Protein concentration related to the cell broth, (B) HA concentration related to the cell broth. Experimental cultivation conditions: Flask volume 250 mL, filling volume 50 mL, modified MS-medium (2.3.3), temperature 26°C, shaking frequency 180 rpm, shaking diameter 5 cm.

Figure 5-7A shows the total soluble protein concentrations of the three different experiments. In the first 48 h of the cultivation, all protein concentrations showed the same progress. After 72 h, the curve of the reference cultivation (20:40) declined and a maximum total soluble protein concentration of 1.1 g/L was obtained after 96 h; by contrast, in the experiments containing 80 mM total nitrogen a maximum protein concentration of about 1.9 g/L was obtained after 96 h. The HA concentration was significantly higher in the cultivations containing 80 mM nitrogen compared to those in the standard MS-medium. A doubled concentration of HA was detected in the 30:50-cultivation and even a quadruple concentration of HA was obtained in the 40:40-cultivation after 168 h (Figure 5-7B). However, a different expression profile could be observed for the two cultivations containing 80 mM initial nitrogen. In the reference cultivation the HA expression went along with the growth of the cells and peaked between 96 and 120 h, whereas in the two cultivations containing 80 mM the HA-expression started after 120 h and peaked at day 7. Here, it would be interesting to measure the HA concentration until day 10 to see if the target protein increased further.
5.4 Discussion

It was evidenced that a mixture of both nitrogen sources is required for an optimal growth of tobacco suspension cultures (Figure 5-1). Additionally, it was proven that two other important factors have to be considered for an optimal nitrogen formulation which is suitable for different plant cell lines:

- the ratio of nitrate to ammonium ions
- the total amount of nitrogen in the medium

For optimal uptake and growth, each plant species requires a different nitrate to ammonium ratio. MUSHARIGE AND SKOOG (1962) reported optimal growth of *N. tabacum* cells at a nitrate/ammonium ratio of 1.91. Suspensions of soy beans grow optimal in the Gamborg B5 medium with a ratio of 12 (GAMBORG ET AL. 1968) and suspension cells of the flower *Haplopappus gracilis* revealed the fastest growth with a ratio of 2.25 (ERIKSSON 1965). In all above mentioned cultivations the nitrate/ammonium ratio is on the side of nitrate. However, it was demonstrated that an ammonium increase and even equimolar concentrations of ammonium and nitrate resulted in clearly higher oxygen transfer rates for BY-2 and NT-1 cells (Figure 5-3 and Figure 5-5). If the modified MS-medium contains more ammonium or if the ratio is in an equimolar balance, the cells do not have to spend extra energy for the reduction of nitrate and therefore, the maximum OTR value is higher (Figure 1-1).

Cultivations in the ammonium-enriched medium (Figure 5-3C and Figure 5-4C) or with an increased initial nitrogen concentration (Figure 5-7A) resulted in higher intracellular protein concentrations compared to cultivations in standard MS-medium. Since ammonium can be used directly as nitrogen-source for protein synthesis, an increase in the initial amount of ammonium led to higher protein concentrations as already shown by GAMBORG AND SHYLUK (1970). A positive effect of the ammonium-enriched medium on the target protein production was shown in Figure 5-3C and Figure 5-4C. Concentrations of both target proteins almost doubled.

During the last 48 h of a cultivation, the HA concentration as well as the inner protein concentration declined. Post-translational protein degradation by proteases along the secretory pathway can reduce the yield of the target protein (DORAN 2006; SCHIERMeyer ET AL. 2005). However, this degradation can be efficiently minimized by an ER-retention, since proteolytic activity is lower in the ER. With ER-retention, a higher protein yield (up to $10^4$ for human epidermal growth factor) has been reported (DORAN 2006) and might also be an explanation why the GFP concentration remained rather stable until the end of the cultivation (Figure
5-3C). However, some proteins require the post-translational modifications in the Golgi for a functional stability (Gomord and Faye 2004) and a protein secreted into the culture broth strongly facilitates further downstream processing (Hellwig et al. 2004).

A recent publication of Holland et al. (2010) demonstrated an up to 20-fold higher concentration of a monoclonal full-size antibody using nitrate-fortified MS-medium in batch fermentation experiments. Here, the MS-medium was enriched with additional 0.1 mol potassium nitrate. Though, the full-size antibody is secreted into the medium and up to now it is not fully understood if the high nitrate concentration in the medium – which is hypertonic to the cells – facilitates the secretion of the protein, has a stabilizing effect on the antibody in the culture broth or has a direct influence on the expression. Similar results have been reported by Sonderquist et al. (2005), where the application of osmotic stress prevented the degradation of the granulocyte-macrophage colony-stimulating factor (GM-CSF) in the supernatant secreted by N. tabacum cells. Related trends have been published for “stabilizers” as bovine serum albumin (BSA) (James and Lee 2001), gelatin (Ryland et al. 2000) and PVP (LaCount et al. 1997). Since in this work recombinant proteins were expressed which are intracellular, the boosting effect of the ammonium-enriched medium can be related solely to the expression of the GFP and consequently stabilizing effects on the recombinant protein can be ruled out.

The presented results clearly proof that a minimum concentration of phosphate and ammonium is required for an unlimited growth of NT-1 cells (Figure 5-5). Upon the given MS-medium composition, the NT-1CsVMVHA cell line became phosphate and ammonium limited. Not until the phosphate concentration was increased to 4.08 mM and the ammonium concentration was increased to 30 mM, an unlimited NT-1 cell growth was observed and the highest biomass concentration of 16.1 g/L was observed (Figure 5-5C and F). As mentioned previously, phosphate plays a pivotal role in cell metabolism and can be a limiting factor for the cell division of tobacco suspension cells if the concentration is too low (Sano et al. 1999). This could be an explanation for the reduced oxygen transfer rates and biomass concentrations with initial phosphate concentrations of 1.25 and 2.04 mM. As higher initial phosphate concentrations supported a faster NT-1 cell growth and higher biomass concentrations, all further experiments with NT-1 cells were performed in phosphate-enriched MS-medium with an initial concentration of 4.08 mM.

In summary, the MS-medium has been improved by the enrichment of ammonium which resulted in a two fold increase of the target protein GFP. Furthermore, this improved medium
was successfully used for another transgenic tobacco cell line to characterize the growth behavior and to boost target product formation of the pharmaceutically relevant protein influenza hemagglutinin (HA). In spite of the media improvement unsolved questions still remain. Why a nearly identical biomass concentration resulted in an OTR of 10 mmol/L/h and an OTR of 6.5 mmol/L/h is not understood, yet (Figure 5-6A and B). Despite significant differences in the highest OTR-values the offline parameter including dry weight, conductivity and pH exhibited a nearly identical progress. Moreover, the trend of the HA concentration is not reproducible. The reason for that is not yet fully understood and must be the subject of further investigations.
Chapter 6 Influence of the osmotic pressure

Plant suspension cells are highly affected by the osmotic pressure of the culture medium (Figure 4-3). Here, four sets of experiments are presented which deal with the influence of an increased osmotic pressure on plant cell growth and protein productivity. In the first one, it was investigated if mannitol as an inert osmolyte can be used to prevent deplasmolysis at the end of the cultivation. In the second experiment, it was tested if ectoine can be used as a compatible solute adapting BY-2 cells to a higher osmotic pressure. In the last two experiments, the influence of different pulse experiments on BY-2 cell growth and recombinant protein production was investigated.

6.1 Influence of mannitol as an inert osmolyte on BY-2 cell growth

During a cultivation cycle, cells experience considerable but steady changes in the osmolality of the MS-medium. Cells are weekly transferred from a hypotonic into a hypertonic medium which could lead to an osmotic shock. Aim of the following experiment was the prevention of deplasmolysis at the end of the cultivation by the addition of mannitol. Moreover, it was investigated if BY-2 cells exhibited a shorter lag phase in the following cultivation cycle by preventing deplasmolysis. For this purpose, the sugar alcohol mannitol was used. Mannitol was added to the culture after 101 h with a final concentration of 0.1 mol/L. For this experiment the BY-2_CaMV GFP-KDEL cell line was used. The influence of the mannitol pulse on cell growth, osmolality and the wet/dry weight ratio over 9 days is shown in Figure 6-1.
Figure 6-1: Influence of a mannitol pulse during a cultivation of BY-2GAVY-GFP-KDEL cells. Solid line represents the data without a pulse and dashed line the data obtained with the addition of the mannitol pulse. Mannitol was pulsed (0.1 Osm/kg) after 101 h and is represented by the dotted line. (A) Oxygen transfer rate (OTR). (B) osmolality and (C) wet/dry weight ratio. Experimental cultivation conditions: Flask volume 250 mL, filling volume 50 mL, temperature 26°C, shaking frequency 180 rpm, shaking diameter 5 cm.

Figure 6-1A represents two OTR-curves obtained accordingly with MS-medium (solid line) and MS-medium exposed with the mannitol pulse (dashed line). Until the pulse, both OTR-curves superimposed. As both cultivations were identical until the mannitol pulse after 101 h, no samples were analyzed in this time period. Just after the mannitol pulse at 101 h, the OTR decreased and reached a 15% lower OTR compared to the reference which could be attributed to an osmotic shock of the BY-2 cells. The osmolality jumped to 0.206 Osm/kg after the mannitol pulse (Figure 6-1B). In the reference culture, the osmolality decreased.
almost to zero resulting in deplasmolysis of the BY-2 cells (Figure 6-1C). The curves of the pH were almost identical (data not shown). The wet/dry weight ratio is an indicator for the progressive deplasmolysis of plant suspension cells. The higher the ratio is the higher the water content of the plant cell. By pulsing mannitol the deplasmolysis of BY-2 cells at the end of the cultivation was reduced. After 9 days of cultivation the wet dry/weight ratio of the reference cultivation was about 20% higher than that of the culture containing mannitol.

In the following experiment it was examined if BY-2 cells coming from this mannitol containing culture show a shorter lag phase in the standard MS-medium compared to BY-2 cells in an advanced level of deplasmolysis. Thus, the experiment was repeated under identical conditions. BY-2 cells coming directly from the RAMOS flasks were used for the inoculation of new flasks. The results are presented in Figure 6-2.
Figure 6-2: Influence of a mannitol pulse in the preculture of BY-2\textsubscript{CsVMV\-GFP-KDEL} cells on cell growth in the main culture. Solid line represents the data without a pulse and dotted line the data obtained with the addition of the mannitol pulse. Mannitol was pulsed (0.1 Osm/kg) after 94 h and is represented by the dotted line. (A) Oxygen transfer rate (OTR), (B) osmolality and (C) wet/dry weight ratio. Experimental cultivation conditions: Flask volume 250 mL, filling volume 50 mL, temperature 26°C, shaking frequency 180 rpm, shaking diameter 5 cm.

The results presented in the first 163 h of the cultivation were similar to the results presented in Figure 6-1. After 163 h the first cultivation was stopped and a new experiment was started in standard MS-medium. Here, BY-2 cells were used which were derived from the cultivation 0 – 163 h (indicated by the black arrow). Thus, also BY-2 cells with a reduced deplasmolysis were used for the inoculation (dashed curves). Figure 6-2A shows that both OTR-curves nearly superimposed. The cells coming from the mannitol containing preculture showed the same lag phase as the cells coming from the reference cultivation. The osmolality was
likewise identical. Only the wet/dry weight ratio directly after the inoculation was doubled, which was probably a measurement error since after 336 h both wet/dry weight ratios were identical. The effect, that mannitol prevents the deplasmolysis was shown, but the desired effect of a shorter lag phase in the next cultivation for BY-2 cells was not detected.

High osmotic pressure induced by mannitol or PEG can increase the production of the secondary metabolite paclitaxel in suspension cultures of *Taxus chinensis*. The osmotic stress could mimic drought stress which can induce secondary metabolism (Kim et al. 2001). A further study demonstrated that an osmotic shock induced by mannitol can enhance recombinant protein secretion of BY-2 cells into the culture medium by accelerated secretion due to a permeabilization of the cell wall (Lee et al. 2002). If this observation could be transferred to the used cell lines is the subject of further investigations. But if the solute concentration is too high, irreparable cell damages might occur. The addition of extremely high solute concentrations results in an osmotic shock to the plant cells causing a rapid change of water movement across its cell membrane. With cell images shown in Figure 6-3 an osmotic shock is illustrated. The osmotic shock was induced by a few drops of a 1.5 M sucrose solution.

![Microscopic images of BY-2 wild type cell aggregates. Osmotic shock as induced by 1.5 M sucrose solution.](image)

**Figure 6-3:** Microscopic images of BY-2 wild type cell aggregates. Osmotic shock as induced by 1.5 M sucrose solution. Experimental cultivation conditions: Flask volume 250 mL, filling volume 50 mL, temperature 26°C, shaking frequency 180 rpm, shaking diameter 5 cm. Light microscopy, 40x magnification.

Figure 6-3A shows BY-2 wild type cells after 72 h of cultivation. The cells are characterized by a dense and structured cytoplasm. By applying the osmotic shock the cells were destroyed and the cytoplasm was drawn out of the cells through osmosis (Figure 6-3B). After the shock most of the plant cells were dead and constricted to cell wall clusters (Figure 6-3C). The previous experiment clearly illustrated that hyperosmotic conditions must be chosen carefully. Hence, in further experiments the osmolality never exceeded 0.3 Osm/kg.
6.2 Hyperosmotic tolerance of *Nicotiana tabacum* BY-2 cells by addition of ectoine

As shown in the previous chapter BY-2 cells are sensitive to high osmotic pressure. NAKAYAMA ET AL. (2000) presented that expressed ectoine conferred hyperosmotic tolerance to transgenic BY-2 cells. Ectoine (1,4,5,6-Tetrahydro-2-methyl-4-pyrimidinecarboxylic acid) was found in *Ectothiorhodospira halochloris*, an extremely halophilic phototrophic eubacterium (GALINSKI ET AL. 1985). It was identified as a compatible solute by acting as an osmolyte. The aim of the following experiment was to examine if low initial concentrations of ectoine confer a hyperosmotic tolerance to transgenic BY-2CsVMVGFP-KDEL cells. Hyperosmotic conditions were created by the addition of NaCl (obtained from a 3 M sterile stock solution) to the MS-medium to a final value of 0.3 Osm/kg.
Figure 6-4: Influence of ectoine and NaCl on cell growth of BY-2<sub>CaVMV</sub>GFP-KDEL cells. (A) Oxygen transfer rates at two different osmolalities, (B) oxygen transfer rates at various initial ectoine concentrations, (C) oxygen transfer rates of various combinations of NaCl and ectoine. Ectoine as well as NaCl were added sterile to the RAMOS flasks to rule out any influence due to the inoculation process. Experimental cultivation conditions: Flask volume 250 mL, filling volume 50 mL, temperature 26°C, shaking frequency 180 rpm, shaking diameter 5 cm.

Figure 6-4A shows the influence of a higher initial NaCl concentration on the OTR of BY-2<sub>CaVMV</sub>GFP-KDEL cells. NaCl was used to artificially increase the initial osmotic pressure to a final osmolality of 0.3 Osm/kg. The reference cultivation showed the growth described previously. The two growth indices, growth rate and the maximum value of the OTR, decreased significantly as initial osmolality increased to 0.3 Osm/kg. This slower growth is in part due to a significant loss of cell viability as cells must cope with high sodium and chloride concentrations. This ion excess might cause toxicity and can therefore, reduce
Influence of osmotic pressure

plant cell metabolism (Greenway and Munn 1980). Figure 6-4B represents the influence of different initial concentration of ectoine. Here, the OTR-curves are strictly arranged in the order of the increasing ectoine concentration, which suggests a negative influence of ectoine on BY-2 cell growth. Furthermore, in this experiment the accuracy of the RAMOS device should also be highlighted. The influence of additives even in the nmol range was still nicely and accurately detected with the RAMOS device. Figure 6-4C represents the influence of the increased osmotic pressure on the OTR in combination with different concentrations of ectoine. It can be noticed that higher osmotic pressure clearly inhibited BY-2 cell growth. The OTR-curves of the experiments containing NaCl superimposed. The effect of the increased NaCl concentration on BY-2 cell growth was predominant in which the effect of ectoine was negligible. Unlike the study of Nakayama et al. (2000), ectoine conferred no hyperosmotic tolerance to the BY-2 cells. In contrast to that study, here, ectoine was added to MS-medium instead of expressed directly by the cells which could be a possible explanation for the obtained results.

By a stepwise, long-lasting adaption to higher salinity, BY-2 cells are able to grow in MS-medium containing up to 35 g/L NaCl (1.19 Osm/kg) (Binzel et al. 1985) and 30% PEG 8000 (Iraji et al. 1989). Besides tobacco other transgenic plant cell suspension cultures have been isolated having enhanced tolerance to higher salinity levels including alfalfa (Croughhan et al. 1978), potato (Salgado-Garciglia et al. 1985) and wheat (Karadimova and Djambova 1993). These suspension cell lines were designed to develop intact plants with a higher salt tolerance. The regeneration of NaCl-tolerant plants from tissue cultures has already been reported for Kickxia ramossisima (Mathur et al. 1980), Datura innoxa (Tyagi et al. 1981) and even tobacco suspension cultures (Nabors et al. 1980).

Conferring hyperosmotic tolerance and adapting plant suspension cells to higher salinities was already in the focus of different research groups mentioned above. These adapted suspension cells have undergone a physiological and a metabolic adjustment which renders them more tolerant to higher extra cellular salt concentrations. Blaschek and Franz (1983) reported a change in the cell wall composition of adapted tobacco cells in response to an increased osmolality of the culture medium. Moreover, the carbon flux of adapted cells is different. Adapted cells may be energy limited due to the diversion of metabolic carbon to other processes which are required for salinity maintenance. Additional carbon is required to accommodate the biosynthesis of osmotic solutes. This resulted in a partitioning of carbon away from growth processes (Hanson and Hitz 1982; Poljakoff-Mayber 1982). Along with the development of salt tolerant plants there is a further utilization of salt adapted
influence of osmotic pressure on suspension cells. Various research groups (SONDERQUIST ET AL. 2005; JAMES AND LEE 2001; LACOUNT ET AL. 1997) found out that the application of osmotic stress prevented the degradation of individual recombinant proteins expressed and secreted by plant suspension cells as it has been discussed in section 5.4. Thus, in some cases it can make sense to adapt plant suspension cells to higher osmotic pressures as these cells have the ability to grow, to secrete and to express their recombinant proteins in the culture broth as they are more stable in a high salinity environment.

6.3 Influence of different nutrient pulses on growth and product formation of a transgenic BY-2 cell line

In this experiment the osmotic pressure was increased by nutrient pulses and not by an inert osmolyte (Chapter 6.1 and 6.2). Three different pulse experiments were carried out: (1) additional 30 g/L sucrose; (2) additional 50% medium; (3) additional 30 g/L sucrose and 50% medium. A higher sucrose concentration was obtained by adding 1.45 mL from a 3 M sterile stock solution. For the addition of the MS-medium 2.21 g were solved in 5 mL, sterilized, and added to the liquid. Aim of these pulse experiments, was to investigate if an addition of further nutrients could enhance the recombinant protein production compared to a reference cultivation without pulse. For this experiment the transgenic BY-2\textsubscript{CmAcl}YFP cell line was used and the fluorescence was detected via 2D fluorescence spectroscopy.
Influence of three different pulse experiments on *Nicotiana tabacum* BY-2CmActYFP cell growth in MS-medium. Black line represents the reference cultivation without pulse, red line represents a 30 g/L sucrose pulse, blue line represents a 50% medium pulse without sucrose and the cyan line represents a 30 g/L sucrose and a 50% medium pulse. The pulse was performed after 100 h and is represented by the dotted line. (A) Oxygen transfer rate, (B) dry weight, (C) osmolality, (D) conductivity, (E) pH-value, (F) ammonium concentration, (G) nitrate concentration, (H) potassium concentration, (I) sucrose concentration, (J) glucose concentration, (K) fructose concentration and (L) total sugar concentration. Experimental cultivation conditions: Flask volume 250 mL, filling volume 50 mL, temperature 26°C, shaking frequency 180 rpm, shaking diameter 5 cm.

The OTR of the reference experiment (black) showed the expected pattern for BY-2 cells under IME conditions (Figure 6-5A) as already shown and described (Figure 4-2). The highest OTR was 7.2 mmol/L/h at 122 h which corresponds to the depletion of the carbon
source (Figure 6-5L). In the sucrose pulse experiment (red), however, the OTR increased further to 9.2 mmol/L/h after 150 h which supports the previous finding. Here, a different limitation must be responsible for the decrease of the OTR since about 25 g/L carbon was still detectable in the medium (Figure 6-5L). The highest OTR-value of the medium pulse experiment (blue) was about 20% lower compared to the reference (Figure 6-5A). Pulsing sucrose and medium (cyan) the OTR increased further to a value of 6 mmol/L/h after 125 h. From here, the OTR decreased continuously to 5.2 mmol/L/h during the last 48 h presumably resulting from an osmotic shock based on the nutrient pulse. Addition of only medium had no influence on the dry weight at which in both experiments with sucrose addition the dry weight increased (Figure 6-5B). Adding only sucrose resulted in a higher dry weight concentration than supplying sucrose and medium which could be attributed to the osmotic shock.

The osmolality increased significantly in all three pulse experiments (Figure 6-5C). The highest value (0.26 Osm/kg) was obtained by pulsing sucrose and medium. After 168 h the measured difference between the reference and the sucrose experiment was 0.07 Osm/kg. The calculated difference was 0.066 Osm/kg differing 5% from the measured value. These findings coincide with the work of YOSHIDA ET AL. (1973) and LAZZERI ET AL. (1988). These authors concluded that there is a high agreement between the calculated and measured osmolality in MS-medium.

The addition of sucrose had no influence on the conductivity as sugar molecules are uncharged. After the addition of medium the conductivity increased slightly above to its initial value of 5.6 mS/cm followed by a continuous decrease to values lower than 3 mS/cm (Figure 6-5D).

After adding fresh medium, the pH dropped down from 5.2 to 4.9 which could be contributed to a resumption of ammonium (Figure 6-5E). Adding only sugar, the pH-value of the culture broth was unaffected. After the medium pulse the ammonium concentration jumped from 3.1 to 13.7 mmol/L followed by a rapid consumption to 1.4 mmol/L within 24 h. In both other experiments ammonium was depleted after about 108 h. The addition of sugar had no significant influence on the nitrate and potassium concentration as their consumption was similar to the consumption of the reference cultivation (Figure 6-5G and H). After the medium pulse a total concentration of 45 mmol/L nitrate was measured in the supernatant (Figure 6-5G). At the end of the cultivation 9 mmol/L nitrate was measured in the medium pulse experiment and 20.3 mmol/L was measured in the experiment where sucrose and medium was added.
The potassium concentration was constant within the first 96 h of the cultivation (Figure 6-5H). Afterwards it decreased to zero in a period of only 48 h. After the medium pulse, the potassium concentration increased to 25.6 mmol/L followed by a similar decrease to final concentrations between 18.4 and 20.2 mmol/L after 168 h. Figure 6-5I – L represent the consumption of the carbon source where the reference cultivation showed the same procedure described as in the previous Chapter (4.1). An addition of only medium had no significant effect on the carbon consumption. In both other experiments where sucrose was added, the concentrations of the two monomers increased due to the further hydrolization of sucrose.

The recombinant protein produced by this cell line is YFP under the control of an actin promoter of *Cucumis melo*. For the detection of the fluorescence of YFP 2D fluorescence spectroscopy was applied (MAROSE ET AL. 1998). Here, the fluorescence is illustrated in so-called contour diagrams where the excitation is plotted over the emission (Figure 6-6). The higher the fluorescence intensities, the brighter the shading in the fluorescence plots. On the left top of the matrix there is an area free of fluorescence. The diagonal area, where emission equals excitation wavelength, represents the scattered light. The spectrum of this transgenic BY-2<sub>CmAct</sub>YFP cell line was subtracted from a BY-2 wild type spectrum to illustrate changes of the fluorescence intensity excluding the auto fluorescence of the BY-2 wild type. In Figure 6-6 only the subtraction spectra are shown.
Figure 6-6: Influence of three different pulse experiments on BY-2\(\text{CmAct}\)YFP formation determined with 2D fluorescence. (A) Fluorescence spectra of day 0 until day 4, (B) fluorescence spectra of day 5-7 obtained at 3 different pulse experiments. The respective pulse was performed after 100 h. 2D fluorescence measuring conditions: 48-well plate, 0.125 g wet weight resuspended in 500 µL supernatant. Excitation: 500 – 550 nm, slit 5 nm, increment 1 nm; Emission: 513-563 nm, slit 5 nm, increment 1 nm. Integration time was fixed by 0.1 ms. Experimental cultivation conditions: Flask volume 250 mL, filling volume 50 mL, temperature 26°C, shaking frequency 180 rpm, shaking diameter 5 cm.
Figure 6-6 represents the subtraction spectra of a BY-2\textsubscript{CmAct} YFP cultivation and the influence of three different pulse experiments on YFP fluorescence intensity. The fluorescence maxima of YFP were $\lambda_{ex}\text{530}/\lambda_{em}\text{545}$ nm. The spectra in Figure 6-6A show an increase of fluorescence intensity of YFP in the first 96 h of cultivation. YFP was produced in the middle of the exponential growth phase, where a high productivity could be observed. In the last three days of the cultivation, the YFP intensity decreased in the reference as well as in all three pulse experiments (Figure 6-6B). In this period, the fluorescence intensities of the reference and the sucrose experiment decreased to their initial value. In the medium pulse experiment, the YFP intensity after 168 h was clearly higher compared to the YFP intensity of the reference. Moreover, the decrease of the YFP intensity was clearly slower. Hence, the addition of further nutrients had a positive influence on the YFP fluorescence. It was not clarified whether the degradation of YFP was prevented or the production of YFP was enhanced. The obtained results revealed that the 2D fluorescence spectroscopy is a suitable method to investigate the production of fluorescence proteins in living cells over time. Furthermore, it was possible to identify influences of different pulse experiments on the YFP intensity.

For bacteria 2D fluorescence is an established tool to follow online metabolic and process parameters of microbial cultures. Due to the possibility of monitoring several fluorophores online, it offers a deep insight into the bioprocess and the cell metabolism (MAROSE ET AL. 1998). For plant cell suspensions, this technique was applied to figure out the viability of BY-2 cells (VAŇKOVÁ ET AL. 2001) and to determine plant cell concentrations as well as the formation of secondary metabolites of *Eschscholtzia californica* and *Catharanthus roseus* (HISIGER AND JOLICOEUR 2005).

Here, the influence of three different pulse experiments on BY-2 cell growth and recombinant protein production were compared to a reference cultivation without a pulse. Cell growth and nutrient consumption of the BY-2\textsubscript{CmAct} YFP cell line (Figure 6-5) was comparable to that of the BY-2 wild type cell line (Figure 3-3) and the BY-2\textsubscript{CsVMV}GFP-KDEL cell line (Figure 4-2). The depletion of the carbon source coincided with the decrease in the OTR (122 h) and at the characteristic shift in the OTR the ammonium concentration was depleted (104 h) (Figure 6-5). The sugar pulse had a strong influence on the biomass formation. In this experiment clearly the highest biomass concentrations with 35 g/L dry weight was obtained (Figure 6-5B). As the sucrose concentration increased, the total amount of accumulated biomass increased too, which is in good agreement with the results of GUO ET AL. (2005). The medium pulse had merely no significant influence on cell growth but rather on the YFP fluorescence intensity. Compared to the reference cultivation, the fluorescence in the experiments where
medium was pulsed was significantly higher. Since ammonium and potassium were depleted in the period from 96 to 120 h this depletion might affect the YFP fluorescence as described in the previous chapter.

Ultimately, these experiments indicated that a nutrient addition enhanced target protein formation based on YFP fluorescence. However, addition of single nutrients by a single pulse resulted in an osmotic shock and might have a negative influence on cell metabolism. For this purpose, a continuous release of nutrients would be beneficial. Thus, the osmotic shock may be avoided and nutrients may be provided to the cells. This effect was analyzed by using the controlled-release system described in the next chapter.

6.4 Influence of controlled-release systems on GFP formation of *Nicotiana tabacum* cells

With the controlled-release technique it is possible simulate fed-batch conditions in shake flasks (JEUDE ET AL. 2006). As described in section (2.8) the applied discs contained ammonium chloride which is released according to a square root of time kinetic. For this purpose, an experiment with the RAMOS device was performed to verify the influence of the controlled-release system on the breathing activity of BY-2*CaMV*GFP-KDEL cells. The aim of this experiment was to investigate if the controlled-release systems were applicable in plant cell suspensions and if a positive effect on BY-2 cell growth and recombinant protein production could be observed.
Figure 6-7: Influence of the initial ammonium concentration on cell growth of BY-2-CsVMV-GFP-KDEL and on the GFP expression. (A) Oxygen transfer rate (OTR), solid: reference; dash: disc containing additional 20 mM ammonium; dash dot-dot: containing initial 40 mM ammonium. Experimental cultivation conditions: Flask volume 250 mL, filling volume 50 mL, temperature 26°C, shaking frequency 180 rpm, shaking diameter 5 cm. Fluorescence was determined via 2D fluorescence: 48 well MTP; 0.1 g wet weight; Excitation: 482 nm; Emission: 502 nm; slit 5 nm; integration time was fixed by 0.1 ms.

Figure 6-7A shows three OTR-curves obtained accordingly with the standard MS-medium (solid line), the ammonium-enriched medium (dash-dotted line) and MS-medium containing an ammonium chloride disk (dashed line). All three OTR-curves exhibited the expected pattern for BY-2 cells under IME conditions as already described. However, the maximum OTR-value was reached at different time points. BY-2 cells cultivated in the ammonium-enriched medium showed a 17 h longer lag phase compared to cells cultivated in the standard MS-medium. The calculated osmolality in the ammonium-enriched MS-medium was 40 mOsm/kg higher compared to that of the standard MS-medium. Since plant cells are highly osmosensitive, induced osmotic stress delayed BY-2 cell growth. Besides the higher initial osmotic pressure, possible detrimental effects of high external chloride ions might also have a negative effect on cell metabolism (FLOWERS ET AL. 1977). Due to the prolonged lag phase, this type of modification was not a favorable option. Considering this disadvantage, a disc containing additional 20 mM ammonium was added to the culture. In the first 72 h of
Influence of osmotic the pressure

cultivation, no differences in the OTR-curves were detected between the reference and the experiment containing a disc. In these two cultivations, the initial osmolality was identical (data not shown) since the additional ammonium chloride is embedded in the silicone matrix. After about 60 h, the OTR of the disc containing culture increased slightly slower compared to the OTR of the reference cultivation. A possible explanation might be that the silicon elastomer material itself had a negative influence on BY-2 cell growth. Experiments with empty (placebo) discs have to be performed in order to investigate whether the silicone material has any detrimental effects on plant cell metabolism. Nonetheless, BY-2 cell growth of the disc containing culture was still faster than that of the culture cultivated in ammonium-enriched MS-medium.

After 120 h of cultivation, the GFP fluorescence was detected offline via 2D fluorescence intensity. Between the reference and the cultivation in the ammonium-enriched medium no significant difference was observed. In the disc containing cultivation, however, the fluorescence signal was about 40% higher.

For the first time the controlled-release system was successfully applied to plant cell culture. Instead of changing the composition of the MS-medium (Chapter 5), here, ammonium was supplied in silicone elastomer discs. As already seen in the previous chapter, additional ammonium enhanced recombinant protein production. This positive effect was also obtained using the controlled-release system. As ammonium has a direct influence on protein synthesis (GAMBORG 1970), it also boosts the recombinant protein production. How, in particular, controlled-release systems affect BY-2 cell growth and GFP formation in detail has to be the subject in an experiment with more offline samples. As the discs were not commercially available only a limited number of discs were provided. Another challenge using these discs is the prevention of contaminations. Here, the discs were washed for 30 s in pure ethanol but still several flasks were contaminated. Other possible sterilization processes are gamma irradiation or e-beam irradiation. However, these processes often have an adverse effect on the integrity of the silicone material and thus direct influence on the nutrient release.

In conclusion, the application of this easy to use diffusion based controlled-release technique is a very promising option to improve media design for plant cells, as initial high osmotic pressures can be avoided and simultaneously additional nutrients can be provided.
Chapter 7  Scale-up of BY-2 cells into stirred tank fermenters

This chapter deals with scale-up experiments from a 250 mL Erlenmeyer flask into a 2 L fermenter. The scale-up was carried out under the assumption of a constant volumetric power input since it is one of the crucial scale-up criteria (SURESH ET AL. 2009). As shake flasks and fermenters have no geometric similarities, it is worthwhile to investigate if under an equal volumetric power input plant cell growth in the fermenter is possible. Although a transgenic cell line (secreting an antibody) was used for these experiments, the focus of this chapter is on BY-2 cell growth characterization and not on the production of the antibody. Moreover, the interrelation of the OTR, effective viscosity and the power input in shake flask was investigated. Finally, the influence of the increasing viscosity on BY-2 cell cultivations was examined.

7.1 Scale-up calculations

The volumetric power input is regarded as one of the crucial values for the scale-up (SURESH ET AL. 2009). The volumetric power input plays a key role in aerobic fermentations performed in shake flasks or stirred tank fermenters. Prior the experiments the volumetric power input in shake flasks at day 0 was calculated. The IME cultivation conditions for shake flasks were used as the basis for this calculation (2.4.2). The geometric conditions of the fermenter are summarized in Table 2-6. Further values which were needed for the calculation are:

- density \( \rho \) 1000 kg/m³
- dynamic viscosity (simplified) \( \eta \) 1 mPa·s
- inner shake flask diameter \( d_s \) 0.085 m

Under the assumption of “in phase” conditions the specific power consumption in shake flasks at low viscosity can be described by the modified power number (BÜCHS ET AL. 2000b):

\[
P'_O = 70 \cdot \text{Re}^{-1} + 25 \cdot \text{Re}^{-0.6} + 1.5 \cdot \text{Re}^{-0.2}
\]

Eq.: 7-1

with \( P'_O \) modified Power number [-] \nRe Reynolds number in shake flasks [-]
The Reynolds number $Re$ can be calculated by means of the equation 7-2.

$$Re = \frac{\rho \cdot n_s \cdot d_S^2}{\eta}$$  \hspace{1cm} \text{Eq.: 7-2}

with

- $Re$: Reynolds number \hspace{1cm} [-]
- $\rho$: density \hspace{1cm} [kg/m³]
- $\eta$: dynamic viscosity \hspace{1cm} [Pa·s]
- $d_S$: inner shake flask diameter \hspace{1cm} [m]
- $n_S$: shaking frequency \hspace{1cm} [1/s]

$$P_0' = \frac{P}{\rho \cdot n_s^3 \cdot d_S^4 \cdot \sqrt{V_{LS}}}$$  \hspace{1cm} \text{Eq.: 7-3}

with

- $P_0'$: modified power number \hspace{1cm} [-]
- $P$: power \hspace{1cm} [W]
- $\rho$: density \hspace{1cm} [kg/m³]
- $n_S$: shaking frequency \hspace{1cm} [1/s]
- $d_S$: inner shake flask diameter \hspace{1cm} [m]
- $V_{LS}$: filling volume shake flask \hspace{1cm} [m³]

By rearranging equation 7-3 to the power $P$ and inserting all parameters, a power of 0.014 W was calculated (BÜCHS ET AL. 2000B). For a filling volume $V_{LS}$ of 0.05 L in the shake flasks a volumetric power input $P/V_{LS}$ of 0.28 kW/m³ was calculated.

With beginning aeration in the fermenter, gas cavities behind the stirrer blades are generated. As a consequence, the flow resistance is reduced and therefore, the power input starts decreasing (BAILEY AND OLLIS 1986). To compensate this reduction the volumetric power input in an unaerated reactor was determined to achieve a volumetric power input of 0.28 kW/m³. For this calculation several methods are published (HENZLER 1982; MICHEL AND MILLER 1962; MÖCKEL ET AL. 1983). In this work the calculation according to Möckel was used:

$$\frac{P_g}{P_0} = \left( 1.384 + \left( \frac{9735 \cdot u_g}{\sqrt{g \cdot D_R}} \right)^2 \right)^{-0.5} + 0.15$$  \hspace{1cm} \text{Eq.: 7-4}

with

- $P_g$: power input \hspace{1cm} [W]
- $P_0$: unaerated power input \hspace{1cm} [W]
- $u_g$: superficial gas velocity \hspace{1cm} [m/s]
For Eq.: 7-4 the superficial gas velocity has to be known. As the cultivation conditions for BY-2 cells in shake flasks and the fermenter should be identical the specific aeration rate at inlet $q_{in}$ was estimated to be 0.25 vvm. The estimation resulted from a correlation between the specific aeration rate in a fermenter and the liquid filling volume of shake flasks (Klöckner and Büchs 2011).

$$u_g = \frac{Q_g}{A_R}$$  \hspace{1cm} \text{Eq.: 7-5}

with $u_g$ superficial gas velocity \hspace{1cm} [m/s]

$Q_g$ volumetric gas flow rate \hspace{1cm} [m³/s]

$A_R$ fermenter cross section area \hspace{1cm} [m²]

The unaerated volumetric power input $P_0/V_{L,R}$ in the fermenter was calculated to be 0.289 kW/m³. With a power number $P_D = 4.9$ for the Rushton turbine in a turbulent flow, the agitation rate could be determined as follows:

$$\frac{P_0}{V_{L,R}} = \frac{P_D \cdot \rho \cdot n_R^3 \cdot d_R^5}{V_{L,R}}$$  \hspace{1cm} \text{Eq.: 7-6}

with $P_0$ unaerated power input \hspace{1cm} [W]

$V_{L,R}$ reactor filling volume \hspace{1cm} [L]

$\rho$ density \hspace{1cm} [kg/m³]

$n_R$ agitation rate \hspace{1cm} [1/min]

$d_R$ blade diameter \hspace{1cm} [m]

To achieve a volumetric power input of 0.289 kW/m³, an agitation rate $n_R$ of 357 rpm was adjusted in all fermentations. This agitation rate is only valid for a single stage stirrer. According to Eq.: 7-2, the Reynolds number $Re$ for the calculated fermentation conditions was determined to be 16730, which indicates a turbulent flow regime.
Table 7-1: Overview of the determined parameter

<table>
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<tr>
<th>Description</th>
<th>Nomenclature</th>
<th>Calculated value</th>
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</thead>
<tbody>
<tr>
<td>Reynolds number in shake flasks</td>
<td>Re_S</td>
<td>21675</td>
</tr>
<tr>
<td>Modified Power number</td>
<td>P_D</td>
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</tr>
<tr>
<td>Power in shake flasks</td>
<td>P_S</td>
<td>0.014 W</td>
</tr>
<tr>
<td>Volumetric power input in shake flasks</td>
<td>P_S/V_L,S</td>
<td>0.28 kW/m³</td>
</tr>
<tr>
<td>Specific aeration rate at fermenter inlet</td>
<td>q_in</td>
<td>0.25 vvm</td>
</tr>
<tr>
<td>Superficial gas velocity</td>
<td>u_g</td>
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</tr>
<tr>
<td>Power in the aerated fermenter</td>
<td>P_R</td>
<td>0.42 W</td>
</tr>
<tr>
<td>Power in the unaerated fermenter</td>
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<td>Volumetric power input in the unaerated fermenter</td>
<td>P_0/V_L,R</td>
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</tr>
<tr>
<td>Agitation rate</td>
<td>n_R</td>
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</tr>
<tr>
<td>Reynolds number in the fermenter</td>
<td>Re_R</td>
<td>16730</td>
</tr>
</tbody>
</table>

7.2 Fermentation of transgenic BY-2 cells

Within this thesis, several fermentations were conducted: Fermentations with three different inoculation densities (3.7, 7.5 and 10%) were performed. Furthermore, the influence of two different antifoam agents (Plurafac® LF1300 and Pluronic® L61) on BY-2 cell growth was investigated. In the experiment with 3.7% inoculation density, no cell growth was observed in the fermenter whereas plant cells grew normally in shake flasks (data not shown). Though it is likely that an inoculation density of 3.7% was below a minimum inoculation density, which is necessary in plant cell fermentations (Jeong et al. 2008). Moreover, the experiments revealed that plant cells reacted sensitively on the antifoam agent. After addition of the antifoam agent Plurafac® LF 1300, respiration activities collapsed and no more cell growth was monitored (data not shown). Therefore, for all fermentations the antifoam agent Pluronic® L61 was used in a final concentration of 0.1 g/L. Three different points should be addressed in the following experiment:

- to compare cell growth of the two transgenic cell lines BY-2_CaMV_GFP-KDEL and BY-2_CaMV_M12
- to investigate the influence of the antifoam agent Pluronic® L61 on plant cell growth in shake flasks
- to compare plant cell growth in shake flasks and in the fermenter under the assumption of an identical volumetric power input

For this experiment the transgenic tobacco cell line BY-2_CaMV_M12 was used for an 8-day cultivation in shake flasks and the fermenter. Since on day 5 the tube for harvesting cells was blocked by dead cells the fermenter data for day 5 are missing. After the fermenter was filled, the same master mix was used for the filling of the shake flasks containing antifoam.
Therefore, both initial values are identical. The parameters of the three individual experiments are shown in Figure 7-1.

**Figure 7-1:** Fermentation of *Nicotiana tabacum* BY-2 CaMV M12 cells in shake flasks and in a fermenter at a constant volumetric power input. Blue represents the data in shake flasks. Green represents the data in shake flask containing antifoam and red correspond to the data obtained in the fermenter. (A) Oxygen transfer rate in shake flasks and the carbon dioxide transfer rate in the fermenter, (B) dry weight, (C) conductivity, (D) osmolality, (E) pH-value, (F) ammonium concentration, (G) effective viscosity, (H) sucrose concentration, (I) glucose concentration, (J) fructose concentration and (K) total sugar concentration. Cultivation conditions: MS-medium, 30 g/L sucrose, 7.5% (v/v) inoculation, temperature 26°C. Experimental conditions shake flask: Flask volume 250 mL, filling volume 50 mL, shaking frequency 180 rpm, shaking diameter 5 cm. Experimental conditions fermenter: Filling volume 1.5 L, stirrer speed 357 rpm, aeration rate 0.25vvm, antifoam concentration 0.1 g/L.
Figure 7-1A shows the two OTR-curves of the shake flask experiments and the CTR measured in the fermenter. Both OTR-curves showed the expected triangle pattern for BY-2 cells described previously (Figure 4-2). Due to a malfunction in the exhaust gas analyzer, the OTR in the fermenter showed unrealistic values and was therefore not shown in the diagram. Based on the results obtained with the RAMOS device it was assumed that the CTR and OTR are equal. In this work, only CTR-values of the fermenter are presented. The CTR of the fermenter increased to 2 mmol/L/h within the first 72 h. After 72 h, the CTR leveled off and plateaus were reached at approximately 2 mmol/L/h until the end of the fermentation indicating an oxygen limitation.

Figure 7-1B represents the biomass concentration represented by the dry weight over time. Initial values of the experiments were nearly identical with 1.25 g/L and 1.4 g/L, respectively. The cultures in shake flasks without antifoam showed an exponential increase and reached the highest dry weight concentration with 14.64 g/L after 96 h. In the shake flasks containing antifoam, a similar maximum value (14.58 g/L) was reached but approximately 22 h later. In the fermenter a linear increase of the dry weight was observed. Here, after 144 h, the highest biomass concentration with 10.31 g/L was measured.

The conductivity decreased in all three experiments in dependence of the respective growth behavior (Figure 7-1C). In both shake flask experiments, the conductivity decreased to almost zero whereas in the fermenter the decrease was significantly slower to a final value of 2.07 mS/cm after 168 h suggesting that there are still nutrients in the culture broth.

Figure 7-1D shows the curves of the osmolalities which were similar to the curves of the conductivity. The only difference was the increase of the curves within the first to 48 h due to the hydrolysis of sucrose. After 48 h, both curves of the shake flask experiments decreased rapidly to final values lower than 10 mOsm/kg. The highest osmolality in the fermenter was 0.23 Osm/kg and was detected after 72 h. Afterwards the curve decreased almost linear to a final value of 0.116 Osm/kg.

In Figure 7-1E the three plots of the pH-values are shown. Although the pH-value was adjusted to 5.8, the measured initial values were 5.16 and 5.01. From the literature it is already known that the pH-value can decrease during autoclaving processes (SKIRVIN ET AL. 1986). In all three experiments, the pH-value varied along values between 4.5 and 6.12. The trend of the two shake flask experiments was similar. After an acidification in the first 24 h, the pH slowly increased to a final value of 6.17 after 187 h. The pH of the fermenter also
passed through the acidification but after 48 h the pH remained stable between 5.1 and 5.3 until the end of the fermentation.

Figure 7-1F shows the ammonium concentration of the three experiments. In shake flasks ammonium was consumed by the cells within the first 96 h. At this time point an ammonium concentration of 1.3 mmol/L was measured in shake flasks containing antifoam and 3.3 mol/L in the fermenter. In the fermenter, ammonium was not consumed by the cells but remained stable at this concentration until the end of the fermentation.

The evolution of the effective viscosities of the three different experiments over fermentation time is shown in Figure 7-1G. Here, the effective viscosity ($\eta_{\text{eff}}$) determined at the effective shear rate ($\gamma_{\text{eff}}$) in shake flasks, according to Peter (2006) is plotted over time. The rheological behavior of the culture broth exhibited a non-Newtonian behavior, in particular a pseudo-plastic behavior (Kato et al. 1978). This behavior is characterized by a decreasing viscosity at an increasing shear rate. In the period of the first 72 h, the effective viscosity slowly increased from its initial value 0.6 mPa·s to 1.5 mPa·s. After 72 h, the effective viscosity in shake flasks jumped rapidly to a maximum value of 152 mPa·s at day six followed by a decrease to an end value of 110 mPa·s after 187 h. In shake flasks containing antifoam, the viscosity showed a similar pattern with a shift of approximately 20 h. In the fermenter the increase of the effective viscosity was significantly slower to a maximum value of 40 mPa·s after 186 h.

Figure 7-1H-K represents the consumption of the carbon sources over fermentation time. Sucrose was completely hydrolyzed into its monomers after 72 h. Both monomers peaked after 72 h at which the glucose concentration was a little lower than fructose. In the shake flasks experiments, no carbon was detected after 120 h. In the fermenter, however, still 7 g/L fructose was measured after 168 h of fermentation.

Figure 7-1 summarized the results of the experiment where BY-2 cell growth in shake flasks was compared to BY-2 growth in the fermenter under the assumption of an identical initial volumetric power input. Furthermore the influence of the antifoam agent Pluronic® L61 on cell growth was examined in shake flasks. These results were obtained with the transgenic BY-2CaMV-M12 cell line with an inoculation density of 7.5%. This transgenic BY-2 cell line was established by the Fraunhofer IME (Aachen) and, thus, had a different origin than the BY-2CaMV-GFP-KDEL cell line. The BY-2CaMV-M12 cell line was characterized by a more homogenous growth building smaller cell cluster and was, therefore, chosen for the scale-up experiments. Nonetheless, both cell lines showed nearly the same growth behavior as already
described in 4.1. However, the highest OTR-value of the M12 cell line was achieved 20 h earlier which could be attributed to the higher inoculation density in this experiment.

The antifoam agent Pluronic® L61 (final concentration 0.1 g/L) had only a minor influence on BY-2 cell growth in shake flasks. BY-2 cell growth (Figure 7-1B) and the corresponding offline parameters (i.e. conductivity and osmolality) were a few hours deferred in comparison to the cultivation without antifoam.

### 7.3 Power input in shake flasks

The experiment described in section 7.2 was repeated under identical cultivation conditions but with an increased inoculation density of 10%. Here, BY-2 cell growth in shake flasks without antifoam was compared to BY-2 cell growth in the fermenter. The offline parameters (dry weight, conductivity, osmolality, pH-value, ammonium concentration, viscosity and carbon source) were also analyzed in this experiment. Between this experiment and the previous one, no significant variations of the respective offline parameters were observed and therefore the data are not shown. In addition to the above listed offline parameters the power input in shake flasks was measured with the power input shaker described by BüCHS ET AL. (2000A). In the following diagram (Figure 7-2), the connections between the OTR, viscosity and the measured power input are illustrated. Moreover, it should be proven that the correlation of PETER (2006) can be used for the calculation of the effective viscosity in shake flasks.

![Diagram](image.png)

**Figure 7-2:** Connection of the oxygen transfer rate, effective viscosity and the volumetric power input in a cultivation of *Nicotiana tabacum* BY-2\textsubscript{CaMV-M12} cells in shake flasks. **Experimental cultivation conditions:** Flask volume 250 mL, filling volume 50 mL, MS-medium, inoculation 10% (v/v), temperature 26°C, shaking frequency 180 rpm, shaking diameter 5 cm.

Figure 7-2 represents the OTR, effective viscosity and the volumetric power input over cultivation time. Closed circles represent the calculated volumetric power input according to BüCHS (2000A). The effective viscosity was calculated (Eq.: 2-78) and with this value the
volumetric power input was determined (Eq.: 7-3). The measured initial volumetric power input was 0.4 kW/m$^3$ and was constant for the first 48 h of cultivation. Thereafter, it increased exponentially (due to an increase in the viscosity) during the cultivation, achieving a maximum value of 2.2 kW/m$^3$ after 113 h of cultivation. Although the viscosity increased until the end of the fermentation, a drop in the volumetric power input was observed, leading to a final value of 1.4 kW/m$^3$. The behavior for this phenomenon is not known.

The calculated values of the volumetric power input were in a good agreement with the measured data (Figure 7-2). Here, the correlation of Peter (2006) was applied for the calculation of the effective viscosity in shake flasks. Only the calculated value at day 7 was approximately 30% lower. The data clearly illustrated that the increase in the power input coincident with the increase in the effective viscosity. This increase took place after the maximum value of the OTR was passed, indicating a low osmolality and low sugar concentrations.

### 7.4 Possible explanations for the oxygen limitation

Under the assumption of an equal volumetric power input in shake flasks and the fermenter, a total different growth behavior was observed. BY-2 cell growth in the fermenter was significantly slower compared to cell growth in shake flasks (Figure 7-1B). A possible reason might be an oxygen limitation in the fermenter. A good indication for that, was the plateau of the CTR after 72 h (Figure 7-1A). High final values of the conductivity and osmolality indicated that by far not all nutrients were consumed by the cells. In this chapter, possible explanations for an oxygen limitation are discussed.

Among various nutrients that are necessary for plant cell growth, such as nitrogen, phosphate and sugar, oxygen is the critical nutrient as it has to be provided continuously, even in batch cultivations. In the case that the specific aeration rate $q_{in}$ is lower than the maximal oxygen transfer rate of the organism, an oxygen limitation occurs that might have a negative effect on plant cell metabolism. Figure 7-3 shows both carbon dioxide transfer rates and the respective dry weight concentrations in the fermenter over time.
For an inoculation density of 7.5% and 10%, the CTR increased to 2 and 2.4 mmol/L/h, respectively. After 71 h (7.5%) and 80 h (10%), the CTR reached a plateau and both CTRs were almost constant at these values until the end of the fermentation (Figure 7-3A). The abrupt change in the breathing activity at 71 h and 80 h indicated a limitation. As all nutrients were sufficiently available at this time point, a nutrient limitation was ruled out. In both fermentations an oxygen limitation occurred which had a strong influence on BY-2 cell growth in the fermenter. After the plateau in the CTR, the increase of the dry weight was linear in contrast to the exponential increase in the shake flasks. An oxygen limitation clearly inhibits plant cell growth. In contrast to the fermenter an exponential increase of the dry weight was observed in the shake flasks (Figure 7-3B) indicating an oxygen limitation. Similar observations have been reported by SCHLATMANN ET AL. (1995) and SHARP AND DORAN (2001).

Considering the initial cultivation conditions in the fermenter (medium, temperature, aeration rate, volumetric power input), this oxygen limitation in the fermenter was unexpected as under identical conditions in shake flasks an unlimited cell growth was observed. That was
guaranteed by the calculations in chapter 7.1. In the following chapters possible reasons for an oxygen limitation in the fermenter are discussed.

### 7.4.1 Possible errors in the scale-up calculation

For an optimal growth of plant cells a sufficient oxygen supply is necessary. A correlation of the specific aeration rate $q_{in}$ of a fermenter for achieving similar head space concentrations in shake flasks is shown in Figure 7-4 (Klöckner and Büchs 2011). This correlation illustrates the relationship between the liquid filling volume of the shake flask $V_{L,S}$ and the specific aeration rate $q_{in}$ in the fermenter.

![Figure 7-4: Correlation for the estimation of the aeration rate $q_{in}$ in the fermenter.](image)

The black curve is valid for 50 mL and 100 mL wide neck as well as 250 mL and 500 mL narrow neck shake flasks. The red curve represents the data of 250 mL, 500 mL, 1000 mL wide neck and for 2000 mL narrow shake flasks. According to this estimation a specific aeration rate of 0.25 vvm was adjusted (indicated by the dotted line in Figure 7-4). This value can only be regarded as a rough estimation but even at reading errors of 25% the resulting aeration rate would be still in the range of 0.2 and 0.35 vvm. Thus, this difference in the specific aeration rate was negligible and was therefore excluded as a possible reason for an oxygen limitation in the fermenter. In stirred tank fermenters specific aeration rates are in the range of 0.05 – 0.1 vvm and in a pneumatic bioreactors specific aeration rates of 0.5 – 1 vvm are applied (Huang and McDonald 2009). However, too high specific aeration rates can inhibit cell growth due to stripping of CO$_2$ and might elicit severe foaming problems (Mirjalili and Linden 1995).
7.4.2 Oxygen limitation in the cell cluster

Plant cells tend to form large cell cluster (Figure 6-3A) due to the secretion of extracellular proteins where the size of the cluster is dependent on the cell line and the cultivation conditions (TATICEK ET AL. 1991). Formation of cell aggregates impacts mass transfer and can lead to inhomogeneities inside large cell aggregates. Cells on the surface of these clusters have a direct contact to the culture broth and therefore, oxygen and nutrients can diffuse in the cells. Cells located in the center of these clusters, however, may become oxygen and nutrient deficient which might result in inferior cell growth (TATICEK ET AL. 1991).

During downstream processing, however, a moderate cell aggregation might be desirable because it enhances sedimentation rates and facilitates media exchange and *in situ* recovery of the culture broth (HUANG AND MCDONALD 2009). Generally, larger cell aggregates enhance mass transfer limitations and resulting in cell damage due to aggregate shattering (NAMDEV AND DUNLOP 1995)

According to KATO ET AL. (1978), *N. tabacum* cells form relatively small cell cluster in the range of 350 – 400 µm. Within this work, there was no significant difference in the size of the cell cluster between aggregates in shake flasks and in the fermenter. Thus, the agglomeration had no impact on the oxygen limitation in the fermenter.

7.4.3 Verification on the onset of gas recirculation

For optimal oxygen supply in the fermenter, the onset of the gas recirculation is necessary. In dependence of the fermenter geometry and the volumetric gas flow rate, the critical agitation rate for a complete gas recirculation below and above the stirrer can be calculated according (NIENOW ET AL. 1979):

$$n_a = 0.6 \cdot g^{0.4} \cdot Q_g^{0.2} \cdot \frac{D_g}{d_R^2}$$  \hspace{1cm} Eq.: 7-7

$$n_b = 2.24 \cdot g^{0.25} \cdot Q_g^{0.5} \cdot \frac{D_R^{0.25}}{d_R^2}$$  \hspace{1cm} Eq.: 7-8

with

- $n_a$  agitation rate at which gas recirculation above the stirrer occurs  \hspace{1cm} [1/s]

- $n_b$  agitation rate at which gas recirculation below the stirrer occurs  \hspace{1cm} [1/s]

- $g$  acceleration due to gravity  \hspace{1cm} [m/s²]

- $Q_g$  volumetric gas flow rate  \hspace{1cm} [m³/s]
The adjusted agitation rate in the fermentations was 357 rpm. According Eq.: 7-7 and Eq.: 7-8, the two critical agitation rates were calculated to be 126 rpm for \( n_b \) and 369 rpm for \( n_a \). Below the stirrer a complete gas recirculation occurred whereas the critical agitation rate \( n_a \) for a gas recirculation above the stirrer was not achieved. But the adjusted agitation rate was only marginally smaller than \( n_a \) and hence, excluded as a possible reason for an oxygen limitation in the fermenter.

### 7.4.4 Influence of an increasing viscosity

One factor that dramatically influences the rheological behavior of a fluid is the viscosity. In the culture broth of BY-2 cells, the viscosity change depends more on the cells themselves as opposed to extracellular proteins (KATO ET AL. 1978). The effective viscosity increased tremendously during the cultivation in the fermenter as well as in shake flasks (Figure 7-1G), and thus, influenced the rheological properties of the culture broth. A similar behavior in the broth viscosity was obtained with Catharanthus roseus suspensions (SCRAGG ET AL. 1986). In this chapter the influence of an increased viscosity on BY-2 cell growth in shake flasks and the fermenter is examined.

In shake flasks an increase in the viscosity can have severe impacts on the flow regime (BÜCHS ET AL. 2000B). In low viscosity fluids the liquid rotates in phase with the movement of the shaker (“in-phase” conditions). In another case, the viscosity increases during the cultivation and the liquid remains at the bottom of the flask instead of rotating with the movement. This state is called “out-of-phase” state and is characterized by a reduced mixing and reduced oxygen transfer (BÜCHS ET AL. 2001; PETER ET AL. 2004). Its occurrence can be determined using the dimensionless Phase number \( Ph \) according to BÜCHS ET AL. (2000B):

\[
Ph = \frac{d_0}{d_s} \cdot [1 + 3 \cdot \log_{10}(Re_f)] > 1.26
\]

**Eq.: 7-9**

<table>
<thead>
<tr>
<th>( d_R )</th>
<th>fermenter diameter</th>
<th>[m]</th>
</tr>
</thead>
<tbody>
<tr>
<td>( d_R )</td>
<td>blade diameter</td>
<td>[m]</td>
</tr>
</tbody>
</table>

| \( Ph \) | Phase number | [-] |
| \( d_0 \) | shaking diameter | [m] |
| \( d_s \) | inner shake flask diameter | [m] |
| \( Re_f \) | Liquid film Reynolds number | [-] |
with the liquid film Reynolds number

\[
Re_f = Re \frac{2}{\pi} \left( 1 - \frac{4}{\pi} \left( \frac{V_{L,S}^{1/3}}{d_s} \right)^2 \right)^2
\]

Eq.: 7-10

with \( Re_f \) liquid film Reynolds number \([-]\)

\( Re \) Reynolds number \([-]\)

\( d_s \) inner shake flask diameter \([m]\)

\( V_{L,S} \) filling volume shake flask \([L]\)

Phase number values > 1.26 indicate that the fluid is “in-phase”; whereas in case of Phase number values < 1.26 the liquid remains on the bottom of the shake flask and is “out-of-phase”. These “in-phase” operating conditions are defined for \( Fr_a > 0.4 \).

\[
Fr_a = \frac{(2 \cdot \pi \cdot n_s) \cdot d_0}{2 \cdot g}
\]

Eq.: 7-11

with \( Fr_a \) Froude number (axial) \([-]\)

\( n_s \) shaking frequency \([1/s]\)

\( d_0 \) shaking diameter \([m]\)

\( g \) gravitational acceleration \([m/s^2]\)

For the given cultivation conditions (2.4.2), the axial Froude number \( Fr_a \) was calculated to be 0.91. As the viscosity increased during the cultivation the danger of reaching “out-of-phase” conditions in shake flasks increased with extended cultivation time (Eq.: 7-10). Figure 7-5 shows the effective viscosity as well as the calculated Reynolds number \( Re \) and the Phase number \( Ph \) in shake flasks over time.
The evolution of the effective viscosity, the calculated Reynolds and the Phase number during an 8-day cultivation in shake flasks is shown in Figure 7-5. Due to increasing biomass concentrations, the viscosity increased during the course of cultivation. A maximum effective viscosity of 152 mPa·s was reached after 139 h. At the end of the cultivation the effective viscosity dropped to a final value of 116 mPa·s. The drop of viscosity might result from autolysis of the cells induced by nutrient starvation at the end of the cultivation (MORIYASU AND OHSUMI 1996). This phenomenon is extensively discussed in Chapter 9.3.

At the beginning of the calculation the initial Reynolds number $Re$ in shake flasks was calculated to be ~25000. In unbaffled shake flasks the critical Reynolds number for a turbulent flow was proposed to be $Re > 60,000$ (PETER ET AL. 2006). Hence, a laminar flow in the shake flask was detected directly from the beginning of the cultivation. After 64 h of cultivation, the Reynolds number dropped quickly and reached similar low values (400 – 600) as detected in the fermenter in the last days of the cultivation. The Phase number decreased from 5.5 to its minimum value of 1.5 within the first 139 h of cultivation. Despite of the immense increase of the effective viscosity (factor 175) the Phase number $Ph$ was permanent above the critical value of 1.26 (marked by the horizontal dotted line) indicating “in-phase” conditions during the entire cultivation. The cultivation with 10% inoculation density showed similar results (data not shown).

As an adequate mixing is essential for a homogenous nutrient and an optimal oxygen distribution, it was investigated if the oxygen limitation could be attributed to a laminar flow regime in the fermenter. For this purpose the Reynolds number of the reactor fluid in the fermenter was calculated over time according the following equation.
Scale-up of BY-2 cells into stirred tank fermenters

\[
\text{Re} = \frac{\rho \cdot n_R \cdot d_R^2}{\eta}
\]

Eq.: 7-12

with

- \( \text{Re} \): Reynolds number in the fermenter  [-]
- \( \rho \): density  [kg/m³]
- \( \eta \): dynamic viscosity  [Pa·s]
- \( d_R \): blade diameter  [m]
- \( n_R \): agitation rate  [1/s]

In this work the effective shear rate in the fermenter was calculated by the Metzner-Otto concept (KRAUME 2003). This concept has been developed for the laminar flow regime in the reactor. Nevertheless it has also been extrapolated to the turbulent flow regime. According to newest findings of HENZLER (2007) the so-called “power concept” is much more reliable to correlate apparent viscosities in the fermenter. Eq.: 7-13 shows the calculation of the viscosity according to the power concept of Henzler:

\[
\eta = \frac{1}{L^2} \cdot \left( \frac{K}{\rho \cdot V_L} \right)^{\frac{2}{m+1}} \cdot \left( \frac{P}{\rho \cdot V_L} \right)^{\frac{1-m}{m+1}}
\]

Eq.: 7-13

with

- \( L \): empirical coefficient  [-]
- \( K \): flow consistency index  [Pa·s^m]
- \( m \): flow behavior index  [-]
- \( \rho \): density  [kg/m³]
- \( P \): power  [W]
- \( V_L \): filling volume  [m³]

Here, the power concept of Henzler could not be applied for the calculation of the apparent viscosity, since not all parameters were available. Figure 7-6 shows the effective viscosity in the fermenter and the calculated Reynolds number \( Re \) of the reactor fluid over time.
Figure 7-6: Effective viscosity and the calculated Reynolds number of a fermentation of *Nicotiana tabacum* BY-2CaVM12 cells. Experimental fermentation conditions: Inoculation density 7.5%, filling volume 1.5 L, stirrer speed 357 rpm, aeration rate 0.25 vvm, antifoam concentration 0.1 g/L. Dash-dotted line represents the critical value of the Reynolds number from a turbulent to a laminar flow.

By considering the initial conditions in the fermenter, the Reynolds number at day 0 of the fermenter $Re$ is 19211 which is larger than $10^4$. Thus, the reactor fluid was turbulent (KRAUME 2003). In the period of the first 20 h the effective viscosity increased to 1.9 mPa·s. The Reynolds number fell below the critical value of $Re \geq 10^4$ (indicated by the horizontal dashed-dotted line) and, thus, the reactor fluid was laminar. Presumably, the fermenter was oxygen limited after 71 h (Figure 7-3A) and, hence, after the change of the flow regime from turbulent to a laminar flow. In order to proof this assumption, a $pO_2$ electrode must be used in further experiments. Within the cultivation the Reynolds number decreased continuously and had a final value of 417 after the 190 h indicating clearly a laminar reactor flow. The fermentation with an inoculation density of 10% showed similar results and was not discussed.

7.5 Discussion

In this work, the feasibility of a scale-up of BY-2 cells from 250 mL shake flasks to a 2 L fermenter was examined based on an a constant volumetric power input in the fermenter. Despite an identical volumetric power input, a different growth behavior was observed in shake flasks and the fermenter. Shake flasks and fermenters are two different types of bioreactors having no geometric similarities. Shake flasks are characterized by a defined gas/liquid mass transfer area. They are surface aerated and the energy is introduced by the vessel wall which is in contact with the rotating liquid. Fermenters, however, are bubble aerated and the energy is introduced by the stirrer.
Since shake flasks are surface aerated, “in-phase” conditions are important to assure oxygen supply and a good mass transfer. During the entire cultivation, the system was “in-phase”, proven by the dimensionless Phase number (Figure 7-5) indicating optimal oxygen and nutrient transfer in the culture broth. The volumetric power input increased with the increasing effective viscosity during cultivation (Figure 7-2). In shake flasks there is maintenance of beneficial cultivation conditions for the system itself. In other words, it is a self-regulating system.

A totally different phenomenon was observed in the fermenter. At the beginning of the fermentation a turbulent flow regime was detected. The Reynolds number is dependent on the viscosity. Figure 7-7 shows a correlation of the Power number $P_O$ as a function of the Reynolds number $Re$ at three different flow regimes.

![Diagram showing the correlation of Power number and Reynolds number](image)

In the turbulent flow regime ($Re > 10^4$), the power number for the Rushton turbine is constant at 4.9 (Figure 7-7). With increasing viscosity the Reynolds number decreased and underran the critical value of $10^4$ (Figure 7-6). Hence, the reactor fluid was laminar and the Power number decreased. In contrast to the shake flasks, the volumetric power input in the fermenter decreased during the cultivation. This contrary evolution of the volumetric power input indicated, that the chosen scale-up criterion was only valid at the beginning of the cultivation.

The experiments illustrated that the scale-up of BY-2 cells from a 250 mL Erlenmeyer flask to the fermenter was not successful. Although volumetric power input is one of the crucial
values for characterizing and upscaling cultures (Suresh et al. 2009) a significantly slower cell growth was observed in the fermenter. Possible explanations are the laminar flow behavior and a reduced volumetric mass transfer coefficient ($k_L\alpha$) due to an increasing viscosity. Since the oxygen transfer seems to be the limiting criterion, a scale-up by keeping the oxygen transfer constant is recommended in further experiments. The $k_L\alpha$-value is also known as a key scale-up criterion (Garcia-Ochoa and Gomez 2009) and has already been successfully applied for an E. coli scale-up from a MTP into a 2 L fermenter (Funke et al. 2010). However, the $k_L\alpha$-value is naturally changing over the course of cultivation due to an altering broth composition, viscosity and oxygen solubility. As the aeration method is different (surface aeration vs. bubble aeration) cell growth differences might also be observed if this criterion is used for the scale-up.
Chapter 8  Scale-down of BY-2 cells into microtiter plates

8.1 Introduction

Cultivations of various microorganisms in microtiter plates (MTPs) are well established in laboratories and companies all over the world. This cultivation platform provides high-throughput at minimal expenses in time, money and work effort. One device which combines high-throughput and online-monitoring is the BioLector (SAMORSKI ET AL. 2005; KENSY ET AL. 2009). The BioLector is a system that can permanently monitor scattered light and, if necessary, the fluorescence of reporter proteins under defined conditions in a MTP during the cultivation. Moreover, other important parameters as the pH-value and the pO$_2$ can also be measured (FUNKE ET AL. 2010). In this work, GFP was used as a reporter protein. To provide a sufficient oxygen transfer to the plant cells, the MTP is continuously shaken. For the monitoring of the GFP formation, light with a defined wave length is sent into each well (excitation), whereas the backscattered light as an indicator for the fluorescence as an indicator for GFP is monitored.

Up to now this device has been successfully used for various organisms such as bacteria and fungi (KENSY ET AL. 2009). However, only few reports on cultivating plant cells in MTPs have been published. Stimulation effects of salts on plant cell growth have been investigated in 24-well plates (KAWANA AND SASAMOTO 2008). According to the literature only one work exists addressing the question if plant cell growth in 48-well plates is possible. GEORGIEV ET AL. (2009), presents in his review results of a preliminary experiment performed at the Chair of Biochemical Engineering (RWTH Aachen University, Aachen). However, a more detailed approach is lacking. Thus, the aim of this thesis was to prove if plant cell growth can be monitored online in MTPs. Moreover, the influence of different modifications of the culture medium on BY-2 cell growth and GFP formation should be examined. Under given cultivation conditions a maximum oxygen transfer capacity ($OTR_{max}$) of 9.6 mmol/L/h was detected via a modified sulfite assay (FUNKE ET AL. 2009). Hence, the plant cells were not oxygen limited.
8.2 Online monitoring of plant cell growth and GFP formation in microtiter plates

In a first experiment, the reproducibility of plant cell growth and recombinant protein production was investigated in the BioLector. For this purpose the transgenic BY-2_{CaMV}GFP-KDEL cell line was used.

![Graph showing scattered light intensity and GFP fluorescence over time.](image)

**Figure 8-1:** Reproducibility of BY-2 cell growth and GFP formation in a 48-MTP. The measurements (scattered light as an indicator for biomass; closed symbols; GFP fluorescence as an indicator for total volumetric yield; open symbols) were conducted in the BioLector with a signal gain factor of 60 for biomass and 100 for GFP. Both curves shown represent the mean values as well as error bars (n = 3) of cultures grown in standard MS-medium. Experimental cultivation conditions: BY-2_{CaMV}GFP-KDEL, 26°C, shaking diameter 3 mm, shaking frequency 900 rpm, filling volume 1000 µL.

Figure 8-1 shows the scattered light intensity as an indicator for the biomass and the fluorescence of GFP intensity over time. Relatively small error bars (n = 3) indicated the precision of the cultivation system and were therefore not shown in the following figures. In this experiment, BY-2 cell growth was significantly slower compared to the growth in shake flasks (see cell growth in Figure 4-2B). The reason for that was not clarified, since BY-2 cells from the same master mix showed faster growth in shake flasks. Moreover, this growth behavior has only occurred once. Further experiments in MTPs showed the typical one-week growth cycle (Figure 8-3).

On the same plate, cell growth of the transgenic cell line was compared to cell growth of the wild type. In addition, the influence of the initial nitrogen concentration on plant cell growth and recombinant protein production was investigated. The results of this experiment are shown in Figure 8-2.
Figure 8-2: Influence of various nitrogen concentrations on BY-2cMV-GFP-KDEL cell growth and GFP production. The measurements (scattered light as an indicator for biomass, panel (A); GFP fluorescence as an indicator for total volumetric yield, panel (B)) were conducted in the BioLector with a signal gain factor of 60 for the biomass and 100 for GFP. Each of the five curves shown represent the mean values (n = 3) of cultures grown in MS-medium containing various nitrogen concentrations. The relative standard deviation of each curve did not exceed ±5%. Reference culture is indicated by the abbreviation Ref. Experimental cultivation conditions: 48-well MTP, 26°C, shaking diameter 3 mm, shaking frequency 900 rpm, filling volume 1000 µL.

Based on the results, the cell growth of the wild type and the transgenic cell line could clearly be differentiated. Moreover, a dependence of the nitrate/ammonium ratio on BY-2 cell growth and GFP intensity was monitored (Figure 8-2). Uninoculated MS-medium served as a control. Here, a slight increase of the scattered light signal was observed. During autoclaving iron phosphate precipitates are formed in the MS-medium (DALTON ET AL. 1983). Due to medium evaporation during cultivation these precipitates were concentrated in the wells and thus, the scattered light signal increased.

In comparison to the transgenic cell line, the wild type showed the fastest growth behavior and reached a slightly higher scattered light intensity (20,000 a.u.) (Figure 8-2A). Both experiments, cultivated in standard MS-medium (circle and up-triangle) showed a similar plot compared to the dry weight concentrations presented in Figure 3-3 and Figure 4-3 suggesting a good correlation between shake flasks and MTPs. Within the first 6 days of the cultivation, no GFP fluorescence of the wild type was detected. After that time period, however, a slight
increase of the GFP intensity was monitored. This increase can be explained by auto-fluorescence of the cells since the increase in the GFP signal coincided with rapid BY-2 cell growth. For the first time, the GFP signal of the BY-2\textsubscript{CsVMV}GFP-KDEL cell line was monitored online over the course of cultivation. After 48 h, an almost linear increase of the GFP signal was monitored for the reference cultivation (20:40; 20.61 mM ammonium and 39.4 mM nitrate) indicating that GFP is considered to be a growth-associated product.

The initial nitrate/ammonium ratio had a direct influence on the scattered light and the GFP intensity (Figure 8-2). In order to verify these results, the experimental approach is identical to that already presented in chapter 5.3.1. The obtained results in the MTP were similar to the results obtained in the RAMOS device (Figure 5-5). Also in the MTP, the experiment with equimolar concentrations of ammonium and nitrate (i.e. 30 mM ammonium and 30 mM nitrate) showed the fastest growth behavior and reached a higher GFP signal within the first 192 h of the cultivation. An increase of the nitrate concentration resulted in inferior cell growth and lower GFP fluorescence. The explanation was already extensively discussed in section 5.3.2.

In a further experiment, the influence of an increased phosphate and ammonium chloride concentration on the growth of the BY-2\textsubscript{CsVMV}GFP-KDEL cell line and its recombinant product formation was studied (Figure 8-3). In contrast to the results presented above, here, the cells showed the expected growth behavior of a one-week experiment.
Scale-down of BY-2 cells into microtiter plates

The influence of different initial phosphate concentrations on BY-2 cell growth (Figure 8-3A) and GFP formation (Figure 8-3C) is shown in Figure 8-3. As phosphate plays an essential role in the cell division of BY-2 cells a reduction of the initial phosphate concentration to 1 mM resulted in slower cell growth (Figure 8-3A) and a lower GFP intensity (Figure 8-3C) (SANO ET AL. 1999). As GFP is a growth-associated product a reduction of phosphate resulted in lower fluorescence intensity, too. Based on these results a minimum concentration of at least 2.7 mM phosphate was demanded for an unlimited BY-2 cell growth. Consequently, a further increase of the initial phosphate concentration up to 7 mM, had no influence on cell growth and recombinant product formation (Figure 8-3A and C).

Figure 8-3B and D show how an increased amount of ammonium chloride affects BY-2 cell growth and GFP formation. In a medium with 4 mM additional ammonium chloride an almost identical growth behavior was observed (Figure 8-3B) compared to the reference cultivation;
though the GFP intensity was a little bit lower (Figure 8-3D). For BY-2 cells growing in medium containing additional 8, 12 and 16 mM ammonium chloride, a slower growth was monitored and a slightly lower scattered light intensity was reached (Figure 8-3B). This result is in line with the findings presented already in Figure 6-7A. The GFP intensity of these three experiments was also lower compared to the reference cultivation. An additional concentration of more than 8 mM ammonium chloride had a negative effect on the metabolism and inhibited plant cell growth, as earlier published (Givan 1979). Interestingly, not until 84 h this growth inhibition occurred. The reason for that is not understood, yet.

In contrast to the experiments where only one offline sample per day was analyzed, it was now possible to monitor BY-2 cell growth and GFP formation continuously online. Thus, a more detailed connection between cell growth and GFP formation was uncovered. In this cell line, the expression of GFP is driven by the constitutive CsVMV promoter, and therefore the expression is tended to be growth-associated (Huang and McDonald 2009; Verdaguer et al. 1998). Surprisingly, the GFP intensity showed no linear increase, but rather constant progress or even a decrease in the period of 96 – 120 h (Figure 8-3C, and D). In this period, the highest increase of the scattered light was observed and thus, a simultaneous increase of the GFP signal was expected. Obviously, GFP was not degraded as the fluorescence showed no significant decrease. One possible explanation for this phenomenon might be the down-regulation of the promoter in this period of the highest growth rate. In order to clarify this phenomenon, a more detailed analysis on the genetic level of the promoter function has to be performed. As this significant increase of the fluorescence signal (Figure 8-3C and D) was not detected in a Western blot (Figure 4-5) measuring effects as the inner filter effect might also distorted the fluorescence signal.

Ultimately, the experiments in the BioLector revealed that BY-2 cell growth in 48-well plates is possible. In addition, it was demonstrated that medium modifications influenced plant cell growth and the GFP production in the same way as shown in shake flasks. However, due to small volumes in the wells (typically < 1000 µL) there are no opportunities for off-line sampling. According to Huang et al. (2009), cultivations in mL-scale might not mimic conditions of stirred large scale processes, especially with regards to the rheological properties (cell aggregation, viscosity). Despite these handicaps, the presented preliminary results show the potential that the combination of high-throughput and high information content makes the BioLector a very powerful tool in bioprocess development.
Chapter 9  Reproducibility and stability of transgenic plant cell lines

9.1 Introduction into maintenance of plant suspension cultures

Up to now, the longtime storage possibility of plant cells is as a callus on an agar plate. For the initiation of a cell suspension culture, callus tissue (approx. 1 cm$^2$) is put into a shake flask containing liquid media and is placed on a shaker. The suspended culture will be formed over the next weeks depending on the plant species and growth conditions. A more detailed description for the establishment of suspension cultures from callus was already published by Mayo et al. (2006).

Suspension cultures are maintained by subculturing the cells regularly in a specific time interval. This interval varies with the cell line, inoculation density, temperature and if the cell line is a wild type or transgenic (Mustafa et al. 2011). Throughout this thesis and by the majority of companies and institutes, plant cells are subcultured by pipetting a determined volume (mL) of a grown suspension into fresh media (2.4.1). This subculturing method is subject to slight variations, as the physiological state of the plant cell culture is not known nor if the same number of cells is transformed into the fresh medium. Thus, the aim of this chapter is to examine the reproducibility of plant cell growth on the basis of the oxygen transfer rate determined with the RAMOS device. Within this work, this device was shown to be a useful and suitable tool in analyzing and comparing plant cell growth (Chapters 3.2 and 4.1).

9.2 Long term investigation of the reproducibility of plant cell growth

On the one hand the reproducibility within the same RAMOS experiment was investigated; on the other hand RAMOS data of almost two years were gathered, compared and evaluated. For all experiments presented in this chapter without exception, IME cultivation conditions (2.4.2) were applied. The experiments were conducted with a wild type as well as with a transgenic cell line. But before the results of almost two years were compared, it was important to investigate how far the OTRs of a completely identical experiment will vary.
Therefore, seven RAMOS flasks were inoculated from the same master mix. Uninoculated medium served as a negative control. The results are shown in Figure 9-1.

![Figure 9-1: Accuracy of the oxygen transfer rate measured with RAMOS within the same experiment. Oxygen transfer rate (OTR) of BY-2 wild type cells with 7 parallel flasks (A) and 4 parallel flasks (B). Experimental cultivation conditions: Flask volume 250 mL, filling volume 50 mL, temperature 26°C shaking frequency 180 rpm, shaking diameter 5 cm.](image)

The OTR of seven identical inoculated RAMOS flasks perfectly superimposed (Figure 9-1A). The maximum deviation of the highest OTR value was lower than 3%. Even in low ranges of 8-10 mmol/L/h the RAMOS device monitored a smooth signal which was absolutely identical for all seven flasks. Additionally, uninoculated medium served as a negative control. Here, no cell growth occurred and hence, the OTR signal was displayed as a smooth signal on the X-axis (dark red curve).

In a different experiment with transgenic BY-2 cells and four identical inoculated RAMOS flasks a similar result was obtained (Figure 9-1B). Here, the maximum deviation was also lower than 3%, and even the tiny change in the breathing activity (after 118 h) of the BY-2 cells was identical in all four flasks. Throughout this work, superimposed OTR-curves were achieved in 56 duplicates, 12 triplicates, and 4 quadruples. Moreover the OTR-curves superimposed even in experiments with five, six and seven identical approaches. For inoculation purposes a master mix of cells and medium was generated. As plant cell
agglomerates are heavy and settle to the ground quickly, this master mix was stirred to assure a homogenous mixture of the cells. While stirring plant cells were removed from the master mix and distributed to the flasks by pipetting. Ultimately, this inoculation method was proven to obtain absolutely reproducible results within one RAMOS experiment.

After this evidence, now the comparability among 19 individual RAMOS experiments in the period of almost two years of research was investigated. For all of these 19 experiments exactly identical inoculation and cultivation conditions were applied (2.4.2). The results are illustrated in Figure 9-2.

Figure 9-2 illustrates 19 OTR-curves of 19 individual RAMOS experiments in a period of 22 months. It can clearly be seen that – although cultivation conditions were identical – a huge variation between the OTR-curves occurred. It should be mentioned, that this culture was the entire time in cultivation and meanwhile not established from callus tissue.

The greater part (75%) of the OTR-curves peaked in the period between 96 and 130 h. But even in this period a difference of more than 23% between the highest OTR-values appeared. In addition, different shapes of the OTR-curves were obtained. Some OTR-curves are characterized by a triangle shape and had a distinctive tip (January 22\textsuperscript{nd}, 2010) and others (March 6\textsuperscript{th}, 2009), however, did not. Surprisingly, two individual OTR-curves exhibited an almost identical progress until 111 h. Afterwards, these two OTR-curves took a different progress indicating a change in BY-2 metabolism.
Based on the huge variability in plant cell growth, it is nearly impossible to compare results obtained from 2009 to results from the year 2011. The risk of drawing premature or even wrong conclusions is very high. Due to this fact, throughout this work, so-called reference cultivations were performed in each experiment. By doing so, changes in plant cell growth could be directly attributed to the change of the cultivation conditions (various media modifications or cultivation conditions) and not to the physiological status of the preculture.

One possibility to increase the reproducibility during subculturing is to add a low concentration (0.0005%) of pectinases to the suspension culture (Dixon 1985). Pectinases break up cell aggregates by dissolving intercellular pectins. Thereby, a reduced aggregate size and hence, a more homogeneous system is obtained (Lee et al. 2004). A further possibility to increase the reproducibility is the application of other subculturing methods. As stated earlier, plant cells are subcultured by pipetting a determined volume (mL) into fresh media. A further possibility for subculturing cells is to collect the cells, determine the fresh weight and inoculate fresh medium with a certain cell mass instead of a certain volume. In the next experiment, the main cultures were inoculated with 15 g/L cell mass. The results are illustrated in Figure 9-3.

Figure 9-3: BY-2<sub>CSVMV</sub>GFP-KDEL cell growth reproducibility inoculated by wet weight. Experimental cultivation conditions: Flask volume 250 mL, filling volume 50 mL, temperature 26°C, shaking frequency 180 rpm, shaking diameter 5 cm.

Figure 9-3 shows 7 OTR-curves of 7 individual RAMOS experiments performed in a period of 10 weeks. Here, the cultures were inoculated with 15 g/L wet weight instead of 5% (v/v) culture broth. The OTR-curves showed by far not the variability as presented in Figure 9-2. Based on the highest OTR-value within this 7 OTR-curves only a deviation of 8 h occurred. Although this experiment covered only a period of approximately 10 weeks, the results evidenced a significantly higher reproducibility using the wet weight-based inoculation method. However, this subculturing method increases the chance of contamination by
additional centrifugation and weighing steps. For a few cell lines, this subculturing method can be applied to enhance the reproducibility, but for academia or industry with usually more than 30 different cell lines this method is not applicable.

9.3 Influence of the physiological status of the preculture on cell growth of the main culture

The physiological status of a preculture is decisive for the cultivation of the main culture (STAFFORD ET AL. 1985). How the cultivation temperature of the preculture affects cell growth of the main culture is shown by the dashed OTR curve in Figure 9-2. This culture was inoculated with cells derived from a 4-week cultivation cycle at 16°C. Due to the reduced temperature the metabolic activity of the cells was lower resulting in a significantly slower growth of the cells in the main culture.

The initial OTR-values varied from 0.2 to 0.5 mmol/L/h (Figure 9-2). In general, the higher the initial OTR the more cells are in the culture. A larger inoculum results in a faster cell growth and an earlier stationary phase (SCHRIPEMA ET AL. 1990). Surprisingly, a higher initial OTR did not automatically resulted in faster cell growth suggesting a different physiological status of each individual preculture.

Up to now, there is no uniform definition of the “right point” of subculturing, e. g. defined PCV or a defined osmolality. The typical subculturing cycle for BY-2 cells is 7-10 days depending on the cultivation conditions (NAGATA ET AL. 1992). How the physiological status of the preculture affects plant cell growth of the main culture is shown in Figure 9-4.
The main cultures were inoculated with cells which were seven (closed symbols, solid line) and four (open symbols, dashed line) days old. The physiological status of the preculture had a significant influence on cell growth in the main culture. Cells coming from the 4 day preculture showed a faster cell growth and reached the highest OTR-value 21 h earlier (Figure 9-4A). Although the initial biomass concentrations were identical (Figure 9-4B) the cells coming from the 4 day preculture had a higher initial OTR-value. Thus, the percentage of living cells in this culture was higher. All other offline parameters in this experiment exhibited the same tendency as the OTR-curve but were shifted approximately 24 h to the left.
indicating a faster nutrient consumption and, therefore, faster cell growth (dashed line; open symbols). But the progression and interconnection of the individual offline parameter have already been discussed extensively in chapter 4.1 and are not focus of this experiment.

After 168 h of cultivation, BY-2 cells are exposed to starving conditions since almost all nutrients are depleted (Figure 4-2). In response to starving conditions various plant cells (tobacco, rice, arabidopsis) begin to auto-digest cell structures and macromolecules and thus, a net protein degradation occurs (CONTENTO ET AL. 2004). This is in line with the findings presented in Figure 5-3C, Figure 5-4C and Figure 5-7A where the inner protein concentration has decreased by more than 50% after the carbon source was depleted. Also in suspension cells of A. thaliana, autophagy was induced by carbon starvation, which triggered afterwards an immediate arrest of cell growth together with a rapid degradation of cellular proteins (ROSE ET AL. 2006). It is known that sucrose starvation induces protease activity and hence, autophagy of BY-2 cell cultures (JOURNET ET AL. 1986; MORIYASU AND OHSUMI 1996). The microscopic pictures (Figure 9-5) prove the clearing of cytoplasmic organelles in BY-2 wild type cells from day 5, 7 and 10.

![Microscopic images of BY-2 wild type cells cultivated under starvation conditions.](image)

An extended cultivation of BY-2 cells in starvation conditions can significantly deplete cellular structures (Figure 9-5). After 120 h, cells were characterized by a dense and structured cytoplasm (Figure 9-5A). After 7 days of cultivation, first cells were empty but still cell aggregates were formed. BY-2 cells which were extended into a long starvation phase were almost all empty and also cell aggregates were broken up (Figure 9-5C).

Conclusively, the physiological status of the preculture has tremendous influence on the main culture. BY-2 cell growth is faster, when the culture is inoculated with cells which are 4 days old as cells have not to re-synthesize their proteins and cell organelles. Holding BY-2 cells under starvation conditions increased the length of the following lag phase. However, a
practical feasibility of this shortened subculturing cycle in academia and industry is doubtable as this method doubles the amount of work for the maintenance of plant cells.

9.4 Stability of a transgenic plant cell line

All aforementioned concerns (9.2) were exclusively attributed to the reproducibility of plant cell growth. Since these long-term studies were performed with a transgenic cell line, a reasonable question was how the recombinant protein (GFP) was affected in a period of 22 months. The results are illustrated in Figure 9-6.

![Figure 9-6](image.jpg)

**Figure 9-6:** Changes of the fluorescence intensity of BY-2_{CasMVGFP-KDEL} cells over time. Experimental cultivation conditions: Flask volume 250 mL, filling volume 50 mL, initial sucrose concentration 30 g/L, temperature 26°C, shaking frequency 180 rpm, shaking diameter 5 cm. Fluorescence was determined in the BioLector: 48 well MTP; 0.1 g wet weight; Excitation: 485 nm; Emission: 520 nm; gain 30.

After almost 100 weeks in suspension, the GFP intensity severely declined to one-third of the initial value (Figure 9-6). Similar results have been reported by Sharp and Doran (2001) and James and Lee (2006) where a hGM-CSF (human granulocyte-macrophage colony-stimulating factor) protein and a mAb (monoclonal antibody) (IgG1) produced by NT-1 cells decreased by more than 80% after 250 subculture cycles; although the NT-1 cell line itself is known to be very stable. The reason of this decrease is the epigenetic transcriptional silencing of transgenes (gene drift) in plant suspension cells that are subcultured for extended time periods (Matzke and Matzke 1998; Phillips et al. 1994). Due to this genetic instability plant suspension cultures will lose the capacity of producing high levels of the protein of interest as cells with suppressed protein expression will dominate the culture.

Consequently, a new transgenic suspension cell line has to be created. Including transformation an establishment of a transgenic suspension culture takes at least 3 months. Among this long time period, the recombinant protein production varies with individual callus lines. Reasons for this variation are the chromosomal part of the insert, the number of insertion events and the occurrence of gene silencing (Mayo et al. 2006). Ultimately, the
genetic instability of plant cell suspensions associated with their heterogeneous suspension cell lines due to somaclonal variation with inconsistent protein expression profiles is a major drawback using plant suspension cultures at industrial scale. Here, the establishment of monoclonal plant suspension cultures with consistent expression levels could be a first breakthrough.

Besides the heterogeneity, the missing possibility to preserve high-producing cell lines by cell banking is a huge issue and a serious disadvantage of plant suspension cells. Although protocols for cryopreservation based on classical controlled freezing rate of BY-2 cell lines have been established (MENGES AND MURRAY 2004; SCHMALE ET AL. 2006) a generic cryopreservation protocol applicable for all plant suspensions is still missing (SCHMALE ET AL. 2006). In contrast to cryopreservation, the co-expression of gene silencing suppressors is discussed as an alternative approach to preserve the high productivity of elite cell lines (BAULCOMBE ET AL. 2000).

For the establishment of cryopreservation protocols the RAMOS device could play a key role as it serves as an ideal tool to monitor plant cell growth in shake flasks. Small changes in the OTR are monitored and can be easily compared to OTRs prior the cryopreservation. The established methodology with OTR-measurements is the origin for further investigations with plant suspension cells in shake flasks.
Chapter 10 Summary and Outlook

10.1 Summary

The objective of this work was a comprehensive characterization of tobacco suspension cultures, in terms of growth, metabolism and recombinant protein production. Thereby, the cultivation of transgenic *Nicotiana tabacum* cells was performed at different scales (shake flasks, fermenter, microtiter plates) ranging from µL- to L-scale. Throughout this work, the RAMOS device, which allows the online monitoring of the oxygen consumption, was proven to be a useful analytic tool, to detect easily online differences of cell metabolism.

Since publications of plant cells cultivated in the RAMOS device are rare, Chapter 3 provided the first overview of cultivations of different tobacco suspension cell lines. Furthermore, two different cultivation conditions applied by the Fraunhofer Institute and the company Dow AgroSciences were compared by using two different tobacco wild type cell lines. By applying higher shaking frequencies (180 rpm instead of 130 rpm) and a slightly higher cultivation temperature (26°C instead of 25°C), the $\text{OTR}_{\text{max}}$ increased by 20%. Hence, the growth rate and the dry weight concentration increased by more than 15%. As a consequence, these cultivation conditions (IME conditions) were applied as standard conditions for all further experiments.

In Chapter 4, a comprehensive characterization of *Nicotiana tabacum* BY-2 cell growth by combining online and offline analysis of multiple parameters is provided for the first time. Six different growth parameters (OTR, wet and dry weight, conductivity, osmolality and pH-value) and 8 different nutrients (sucrose, glucose, fructose, nitrate, ammonium, phosphate, potassium and sulphate) were analyzed in biological triplicates every 24 h over a cultivation time of 240 h. Moreover, the concentration of the total soluble protein and the recombinant protein GFP was measured. Here, an online detection of the ammonium depletion based on the OTR was shown. A more detailed analysis of the nutrients identified the depletion of ammonium as the reason for a change in the breathing activity.

On the basis of the results presented in Chapter 4, Chapter 5 illustrates a media optimization of the commercial MS-medium. A significant influence of the initial ammonium concentration was demonstrated. The MS-medium has been successfully reproduced from
stock solutions in order to modify initial concentrations of single nutrients. The addition of extra ammonium resulted in a two fold increase of the GFP concentration. Furthermore, this improved medium was successfully used for another transgenic tobacco cell line to characterize the growth behaviour and to boost target product formation of the pharmaceutically relevant protein influenza hemagglutinin (HA).

Since plant cells are highly osmosensitive, the osmotic pressure is a crucial parameter for cultivating plant suspension cells. Chapter 6 describes experiments regarding the influence of increased osmotic pressure in plant suspension cells. The osmotic pressure was artificially increased by using the sugar alcohol mannitol. The addition of mannitol clearly reduced the wet/dry weight ratio of the culture. For the first time, the influence of the so called controlled-release system was applied for plant suspension cultures. This allows the supplementation of more nutrients without a simultaneous increase of the initial osmotic pressure. The suitability of this system was proven for plant cells for the first time. Furthermore, a higher fluorescence intensity was achieved by using controlled-release systems.

Besides in shake flasks, plant cells were also cultivated in a stirred tank reactor. As a scale-up criterion, a constant volumetric power input was chosen. In shake flasks a volumetric power input of 0.28 kW/m³ was determined. Applying this value as the initial volumetric power input in the fermenter, a reduced BY-2 cell growth was observed. However, an unlimited cell growth was observed in shake flask. Here, the volumetric power input increased over the cultivation time and, therefore, the scale-up criterion was no longer given.

In addition, cultivation experiments in MTPs were performed with BY-2 cells. These experiments were conducted in the BioLector. A device allowing the online detection of important fermentation parameters (scattered light, GFP). The experiments discussed in Chapter 8 demonstrate the possibility of plant cell cultivations in 48-well MTPs. Results obtained in shake flasks with modified media could nicely be transferred to MTPs. Thus, the feasibility of cultivating plant cells in µL-scale was successfully proven.

Finally, the reproducibility of plant cell growth in shake flasks was evaluated by comparing 19 individual OTR-curves obtained in a period of 22 months (Chapter 9). Furthermore, in the same period, the stability of the recombinant protein production (GFP) was investigated. In a period of 22 months the GFP concentration decreased by more than 80%. Based on the highest OTR-value, a growth difference of almost 48 h was observed in a 7-day experiment. By changing the inoculation method the reproducibility was strongly increased.
10.2 Outlook

A detailed characterization of a transgenic *N. tabacum* cell line was presented in this thesis. For the first time, this cell line was cultivated in the RAMOS device. The comprehensive analysis of online and multiple offline parameters were shown to be a powerful tool for the characterization of plant suspension cultures. Prospective applications of this concept are the analysis of further plant suspension cell lines (i.e. rice, soybeans or corn) as these cells are also a platform for producing pharmaceutically relevant proteins. The development of an optimal formulation of a basal salt mixture for each individual plant suspension culture is elusive as the medium has to be adapted to each individual cell line. Especially here, the use of the RAMOS device is highly beneficial as nutrient depletions can be easily detected online. One the one hand a properly optimized medium can reduce the process costs as the initial price/L can be reduced; on the other hand the production of the target protein can be enhanced.

As plant suspension cells are highly osmosensitive, high initial osmotic pressures have to be avoided. In further investigations, the controlled-release system should be used and special attention should be paid to a more frequented sampling. Moreover, placebo discs have to be used to investigate the influence of the elastomer itself on plant cell metabolism. Avoiding high initial osmotic pressures and supplying simultaneously additional nutrients can open a new door in media optimization as every nutrient can be embedded in the discs. In addition, new feeding strategies can be investigated which might be also important for new process strategies in fermenters (fed-batch).

Extensive research over the past 20 years has illustrated that plant suspension cells are an established production platform for pharmaceutically relevant proteins. Certainly plant-made pharmaceuticals offers advantages over mammalian cell culture due to lower medium costs and a good scale-up potential. Currently, some of these proteins reach advanced levels of clinical trials. However, two large hurdles remain to be a strong competitor to mammalian cell culture: (1) no possibility to preserve elite cell lines by cryopreservation; (2) the genetic instability of plant suspension cultures. Without the possibility to obtain well defined starting material for each individual fermentation process, plant suspension cells will never be an option for economically relevant large scale processes. In this work, this well-known problem was analyzed in detail by using the RAMOS device and summarized in one diagram. The genetic instability is the second serious problem handling plant suspension cultures. Based on somaclonal variation or transcriptional gene silencing cultures will lose the capacity for a high production of the protein of interest. Here, the establishment of monoclonal plant cell cultures
would be a further milestone for the production of PMPs. Overcoming these two bottlenecks, plant suspension cells might be an alternative platform for the production of therapeutically important proteins as plant cells do not carry potentially harmful human or animal viruses into the PMP. The established methodology presented in this work serves as an origin for further optimizations of plant cell suspension cells.
References


Cardineau GA, Manson HS, VanEck JM, Kirk DD, Walmsley AM; 2009. Vectors and cells for preparing immunoprotective compositions derived from transgenic plants. USA.


## Appendices

### A.1 List of chemicals

<table>
<thead>
<tr>
<th>Chemical</th>
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<td>Potassium hydroxide</td>
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<tr>
<td>Potassium iodide</td>
<td>Sigma Aldrich GmbH</td>
</tr>
<tr>
<td>Potassium nitrate</td>
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<tr>
<td>Potassium sulfate</td>
<td>Merck KGaA</td>
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<tr>
<td>Pluronic L61</td>
<td>BASF</td>
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<td>Sodium chloride</td>
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<tr>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>Sodium molybdate</td>
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<tr>
<td>Sucrose</td>
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<tr>
<td>Nitric acid</td>
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<td>----------------</td>
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<tr>
<td>Thiamine-HCl</td>
<td>Merck KGaA</td>
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<tr>
<td>Tris-HCl</td>
<td>Merck KGaA</td>
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<tr>
<td>Tween 20</td>
<td>Merck KGaA</td>
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<td>Zinc sulfate</td>
<td>Sigma Aldrich GmbH</td>
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### A. II List of consumables

<table>
<thead>
<tr>
<th>Device</th>
<th>Company</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuracy weighing machine</td>
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<td>Göttingen</td>
</tr>
<tr>
<td>Autoclave Systec</td>
<td>Systec Controls</td>
<td>Puchheim</td>
</tr>
<tr>
<td>Autoclave CertoClav</td>
<td>KELOMAT Sterilizer-Devision</td>
<td>Traun, A</td>
</tr>
<tr>
<td>BIOSTAT BiPlus-Fermenter</td>
<td>Sartorius AG</td>
<td>Göttingen</td>
</tr>
<tr>
<td>Blot-Module</td>
<td>Invitrogen GmbH</td>
<td>Eggstein</td>
</tr>
<tr>
<td>Büchner funnel</td>
<td>Thermo Fischer Scientific</td>
<td>Bremen</td>
</tr>
<tr>
<td>Centrifuge Allegra X-15R</td>
<td>Beckman Coulter GmbH</td>
<td>Krefeld</td>
</tr>
<tr>
<td>Centrifuge Rotina 35 R</td>
<td>Andreas Hettich GmbH &amp; Co KG, D</td>
<td>Tuttlingen</td>
</tr>
<tr>
<td>Centrifuge Sigma 1-15 K</td>
<td>SIGMA Zentrifugen GmbH</td>
<td>Osterode</td>
</tr>
<tr>
<td>Clean bench LaminAir</td>
<td>Heraeus Instruments GmbH</td>
<td>Bad Grund</td>
</tr>
<tr>
<td>Conductometer LF 340-A</td>
<td>WTW Weilheim</td>
<td>Weilheim</td>
</tr>
<tr>
<td>Digital camera Cyber Shot N2</td>
<td>Sony Corp</td>
<td>Tokyo, JPN</td>
</tr>
<tr>
<td>Desiccator</td>
<td>Schott AG</td>
<td>Mainz</td>
</tr>
<tr>
<td>Drying chamber</td>
<td>Viessmann GmbH</td>
<td>Hof</td>
</tr>
<tr>
<td>Erlenmeyer flask 250mL, narrow neck</td>
<td>Schott AG</td>
<td>Mainz</td>
</tr>
<tr>
<td>Exhaust gas analyzer NGA 2000</td>
<td>Fischer-Rosemount GmbH</td>
<td>Hasselroth</td>
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<tr>
<td>HPLC Detector RI-101</td>
<td>Shodex Europe</td>
<td>München</td>
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<tr>
<td>HPLC Column PB 2+</td>
<td>CS-Chromatographie</td>
<td>Langerwehe</td>
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<tr>
<td>HPLC System UltiMate3000</td>
<td>Dionex Corporation</td>
<td>Sunnyvale, USA</td>
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<tr>
<td>Infors shaker HAT</td>
<td>Infors</td>
<td>Bottmingen, CH</td>
</tr>
<tr>
<td>Kühner shaker</td>
<td>Kühner AG</td>
<td>Birchfelden, CH</td>
</tr>
<tr>
<td>Magnetic stirrer Big Squid</td>
<td>IKA Werke</td>
<td>Staufen</td>
</tr>
<tr>
<td>Microscope Eclipse E600</td>
<td>Nikon</td>
<td>Tokyo, JPN</td>
</tr>
<tr>
<td>MTP reader Synergy 4</td>
<td>BioTek GmbH</td>
<td>Bad Friedrichshall</td>
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<tr>
<td>Osmometer Osmomat 030</td>
<td>Gonotec GmbH</td>
<td>Berlin</td>
</tr>
<tr>
<td>pH-meter pH510</td>
<td>Euchtech Instruments</td>
<td>Simi Valley, AUS</td>
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<tr>
<td>Photometer Spektroquant NOVA 60</td>
<td>Merck KGaA</td>
<td>Darmstadt</td>
</tr>
<tr>
<td>RAMOS device</td>
<td>AVT.BioVT</td>
<td>Aachen</td>
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<tr>
<td>Rheometer Physica MCR 301</td>
<td>Anton Paar GmbH</td>
<td>Ostfildern</td>
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<tr>
<td>ScannerPerfection V700 Photo</td>
<td>Epson</td>
<td>Tokyo, JPN</td>
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<tr>
<td>Ultrasonic LabsonicU</td>
<td>B. Braun Biotech International GmbH</td>
<td>Melsungen</td>
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<tr>
<td>Vacuum bottle</td>
<td>Schott AG</td>
<td>Mainz</td>
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<tr>
<td>Vacuum pump No. 26.1.2. A.N.18</td>
<td>KNF Neuberger</td>
<td>Freiburg i. Br.</td>
</tr>
<tr>
<td>Weighing machine</td>
<td>Kern &amp; Sohn GmbH</td>
<td>Balingen</td>
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<tr>
<td>Xcell SureLock Mini-Cell</td>
<td>Invitrogen GmbH</td>
<td>Eggstein</td>
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</table>
## A. III List of devices

<table>
<thead>
<tr>
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<th>Company</th>
<th>Location</th>
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<td>Desiccator</td>
<td>Schott AG</td>
<td>Mainz</td>
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<tr>
<td>Drying chamber</td>
<td>Viessmann GmbH</td>
<td>Hof</td>
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<tr>
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<td>DowAgrosciences</td>
<td>Indianapolis, USA</td>
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<tr>
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<tr>
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<td>Invitrogen GmbH</td>
<td>Eggstein</td>
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### A. IV Detailed composition of the MS-medium

Table A.IV: Medium formulation of the MS-medium according Musharige and Skoog (1962)

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<th>Ingredient</th>
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<td>KNO$_3$</td>
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<td>CaCl$_2$•2H$_2$O</td>
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<td>KH$_2$PO$_4$</td>
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<td>CuSO$_4$•5H$_2$O</td>
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</tr>
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<td>CoCl$_2$•6H$_2$O</td>
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<td>FeSO$_4$•7H$_2$O</td>
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<td>Na$_2$EDTA•2H$_2$O</td>
<td>37.3</td>
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A) Aspects of this thesis have been published/presented previously:


B) Contributions to associated publications during this thesis