TAMPEREEN TEKNILLINEN YLIOPISTO
TAMPERE UNIVERSITY OF TECHNOLOGY

ELVIIRA KÄRKKÄINEN

PROPAGATION OF PLANT CELLS IN ROBUST SINGLE-USE BIOREACTORS

Master of Science Thesis

Examiner: Assistant professor Ville Santala
Examiners and topic approved by the Faculty Council of the Faculty of Natural Sciences on the 8th of August 2018

ABSTRACT

ELVIIRA KÄRKKÄINEN:
Propagation of plant cells in robust single-use bioreactors
Tampere University of Technology
Master of Science Thesis, 77 pages, 3 Appendix pages
September 2018
Master's Degree Program in Biotechnology
Major: Bioengineering
Examiner: Assistant professor Ville Santala

Keywords: plant cell, lingonberry cell culture, bioreactor, disposable bioreactor, future food production

New and effective technologies are needed to full fill the increasing demands of sustainable food production due to the growing population, urbanization, climate change, and decreasing water supplies. Plant cell cultivation in closed bioreactor systems offers an attractive new approach for this challenge. Innovation about the production of plant cells in the non-laboratory environment has been studied in VTT Technical Research Centre of Finland. This thesis continued the development of home bioreactor for the robust propagation of plant cells as a food source.

The first aim of this study was to find out which are the key physical factors affecting the biomass accumulation of lingonberry cell suspension culture. Multiple growing experiments under shake flask scale and 2 L working volume stirred tank cultivations were carried out to get more detailed data about growth parameters of lingonberry cell culture. For effective growth without long lag phase inoculum density should be above 60 g/L. Lingonberry cell's need of dissolved oxygen is surprisingly moderate, optimal target would be 5-10 %. To maintain sufficient gas exchange level and prevent cell sedimentation, gentle but effective mixing is needed. The light had a most remarkable impact on biomass accumulation. Cells cultivated in dark produced 47 % more fresh biomass compared to cells grown under white light with 16:8 h photoperiod. Pigment formation revealed to be reversible and dependent on sufficient amount of light.

All previous trials for lingonberry cell cultivations in simple single-use bags have been failed. Gained new information about physical growth demands from this study made designing of suitable single-use bioreactor now possible. Based on the results optimal and as simple as possible single-use bioreactor prototype and cultivation bag were designed. The bag in a box -solution with orbital shaking was chosen. In this model mixing and aeration can be introduced in a simple way without sparging and risk of foaming. Lingonberry cells were successfully cultivated in single-use cultivation bag and biomass accumulation reached promising levels (174 g/L) compared to shake flask (208 g/L) and stirred tank bioreactor cultivations (210 g/L).

Based on all the results of this study, lingonberry cell suspension cultures can now be cultivated in a simple single-use bioreactor. Development of robust home usable bioreactor for plant cell production can be continued and future decisions related to prototype design and building can be based on solid data. However, to achieve optimal biomass accumulation, more studies related to process optimization, especially in lighting and mixing conditions, should be carried out with the designed single-use bioreactor.

TIIVISTELMÄ

ELVIIRA KÄRKKÄINEN:
Kasvisolujen tuotto vakaissa kertakäyttöbioreaktoreissa
Tampereen teknillinen yliopisto
Diplomityö, 77 sivua, 3 liitesivua
Syyskuu 2018
Biotekniikan diplomi-insinöörin tutkinto-ohjelma
Pääaine: Bioengineering
Tarkastaja: apulaisprofessori Ville Santala
Avainsanat: kasvisolu, puolukan soluviljelmä, bioreaktori, kertakäyttöbioreaktori, tulevaisuuden ruuan tuotanto

Väestön kasvaessa, yhteiskunnan kaupungistuessa, ilmaston muuttuessa ja vesivarantojen vähetessä kestävä ruuan tuotanto tarvitsee kipeästi uusia ja tehokkaita teknologioita. Kasvisolujen kasvatus bioreaktoreissa tarjoaa tähän uuden ja mielenkiintoisen lähestymistavan suljetuissa olosuhteissa. Valtio tieteellinen tutkimuskeskus VTT on tutkinut innovaatiota kasvisolujen tuottamisesta ei-laboratorio ympäristössä. Tämä diplomityö jatkaa kotikäyttöisen bioreaktorin kehitystä kasvisolujen kasvattamiseksi ruuan tuotantoa varten

Työn ensimmäisenä tavoitteena oli selvittää puolukan solukasvatuksen biomassan tuottoon vaikuttavia fysikaalisia tekijöitä. Yksityiskohtaisen kasvuparametridatan saamiseksi suoritettiin useita kokeita kasvatuspulloista aina 2 litran bioreaktori kasvatuksiin. Kerätyn datan perusteella voidaan todeta, että tehokkaan kasvun saavuttamiseksi ilman pitkää viive vaihetta inokulaatin pitoisuuden tulisi olla yli 60 g/L. Liuenneen hapen tarve on yllättävän maltillinen, 5-10%. Riittävän kaasujenvaihdon ja solujen laskeutumisen estämiseksi tarvitaan hellää mutta riittävän tehokasta sekoitusta. Biomassan tuoton kannalta merkittävin fysikaalinen tekijä näytti kuitenkin olevan valo. Pimeässä kasvatetut solut saavuttivat kokeissa jopa 47 % korkeamman biomassan, kuin solut, jotka kasvoivat valkoisessa valossa 16:8 h valojaksolla. Puolukan solujen pigmentin muodostus paljastui olevan palautuvaa ja riippuvaista riittävästä valon määrästä.

Puolukan solujen kaikki tätä työtä edeltäneet kasvatusyritykset yksinkertaisissa kertakäyttökasvatuspusseissa olivat epäonnistuneet. Saatu tieto solujen fysikaalisista kasvuvaatimuksista teki sopivan kertakäyttöbioreaktorin suunnittelusta kuitenkin nyt mahdollista. Tulosten pohjalta suunniteltiin mahdollisemman optimaalinen ja yksinkertainen kertakäyttöisen bioreaktorin ja kasvatuspussin prototyyppi. Sovellukseen valittiin bag in a box -tekniikka orbitaalisella sekoituksella. Kyseisessä mallissa ilmastus ja sekoitus voidaan toteuttaa yksinkertaisesti ilman kuplitusta, ja siten kasvatuksen vaahtoamisriskiä. Puolukan soluja onnistuttiin kasvattamaan suunnitellussa yksinkertaisessa bioreaktorissa suhteellisen hyvällä biomassan tuotolla (174 g/L) verrattuna niin kasvatuspullo (208 g/L) kuin stirred tank -bioreaktori kasvatuksiin (210 g/L).

Tutkimuksessa saatujen tulosten ansiosta puolukan soluja voidaan nyt kasvattaa yksinkertaisissa kertakäyttöbioreaktoreissa. Vakaan kotibioreaktorin kehitystyötä voidaan jatkaa tämän pohjalta, ja tulevaisuudessa prototyypin suunnitteluun ja toteutukseen liittyvät päätökset voidaan perustaa luotettavaan mittausdataan. Saaduista tuloksista huolimatta, optimaalisen biomassan tuoton saavuttamiseksi lisää tutkimusta prosessin optimoinnista, etenkin valon ja sekoituksen kannalta suunnitellussa kertakäyttö-bioreaktorissa tarvitaan yhä.

PREFACE

This master's thesis work was conducted in the Plant biotechnology team in VTT Technical Research Centre of Finland at Espoo from February 2018 to April 2018.

I am truly thankful for my supervisors Heiko Rischer and Lauri Reuter, who made this inspiring opportunity to carry out my master's thesis work under interesting and topical Home-bioreactor -project possible for me. It has been really motivating to work with a project which I followed from media with high interest already years before knowing that at some point I would be also part of this fascinating study. Thank you for the guidance, believe and support during the process and the change of being part of the research group trying to solve the challenges of novel food production in a very innovative way! It has been my pleasure.

I would like to thank all my team members about a warm and welcoming attitude. I really enjoyed my time at the lab and coffee breaks with you! Special thanks to Jaana Rikkinen, lab-mom and saving angel of every student, you are amazing. I would also like to say many thanks for Toni Paasikallio about your guidance of the secrets of pilot hall and tricks of bioreactors.

Many thanks also to my supervisor Ville Santala from your valuable feedback and comments.

And finally, I would like to thank all my friends for the great support not only during my thesis project but throughout my whole university years. Special thanks to people from UrOs and Bioner from all the immemorial moments. Because of you guys, my years at TTY were the best time of my life! Above all, thanks to you Joel for encouraging me and for pushing me out of my comfort zone always when I needed it. I cannot even thank you enough for all your support.

Helsinki, 16.9.2018

Elviira Kärkkäinen

TABLE OF CONTENTS

1. INTRODUCTION 1
2. THEORETICAL BACKGROUND 3
2.1 Plant cell products 3
2.2 Plant cell suspension cultures 6
2.2.1 Features and growth of plant cells 7
2.2.2 Oxygen demand 8
2.2.3 Shear stress sensitivity 8
2.2.4 Foaming and wall growth 9
2.2.5 Temperature and pH 9
2.2.6 Secondary metabolite formation 10
2.3 Plant cell cultivation in bioreactors 10
2.3.1 Process optimization to increase the growth of plant cells 11
2.3.2 Aeration and dissolved oxygen 12
2.3.3 Agitation 13
2.3.4 Inoculum density and age 14
2.3.5 Light conditions 15
2.4 Disposable sigle-use bioreactors 16
2.4.1 Benefits and limitation of disposable bioreactors 17
2.4.2 Wave bioreactor 20
2.4.3 Stirred bag bioreactor 20
2.4.4 Pneumatically mixed bioreactor 21
2.4.5 Orbitally shaken bioreactor 21
2.4.6 Multi-chamber bioreactor 22
2.5 Home bioreactor 23
3. MATERIALS AND METHODS 25
3.1 Used strain, medium and culture conditions 25
3.2 Shake flask experiments 26
3.2.1 Comparison growth curves of tobacco NY-2 and lingonberry cell cultures 27
3.2.2 Inoculum density experiment 27
3.2.3 Gas exchange experiment 27
3.2.4 Dark cultivation experiment 27
3.2.5 Two-stage cultivation experiment 28
3.3 Wave bioreactor cultivation of lingonberry cell culture 28
3.4 Stirred tank bioreactor experiments of lingonberry cell culture 28
3.4.1 Lighting 29
3.4.2 Inoculation of stirred tank bioreactors 30
3.4.3 Culture conditions 30
3.4.4 Sampling and measurements 33
3.5 Sugar consumption analysis 33

3.6 Anthocyanin content analysis....33
3.7 Viability measurement and cell morphology....34
3.8 Single-use bag experiments of lingonberry cell culture....34
3.9 Statistics....37
4. RESULTS....38
4.1 Determination of base-line growth....38
4.1.1 Inoculum density....39
4.1.2 Gas exchange....42
4.1.3 Light conditions....42
4.2 Stirred tank bioreactor cultivations of lingonberry cell culture....46
4.2.1 Inoculum strategy....50
4.2.2 Critical oxygen levels....50
4.2.3 Sugar consumption....52
4.2.4 Pigment formation, anthocyanin content and visual appearance....52
4.2.5 Air-lift approach....54
4.3 Disposable bioreactor cultivations of lingonberry cell cultures....55
4.3.1 Wave bioreactor....55
4.3.2 Single use cultivation bag....56
5. DISCUSSION....60
5.1 Physical key factors for biomass production....60
5.2 Single-use cultivation bag and Home bioreactor prototype....63
6. CONCLUSIONS....66
7. REFERENCES....69

APPENDIX A: CHEMICAL COMPOSITION OF MS MEDIUM -POWDER

APPENDIX B: CHEMICAL COMPOSITION OF MCCOWN WOODY PLANT INCLUDING VITAMINS -POWDER

APPENDIX C: VIABILITY DATA

TERMS AND DEFINITIONS

BEH	Ethylene Bridged Hybrid particle/technology
BY-2	Bright Yellow 2
CO_2	Carbon dioxide
C_2H_4	Ethylene
CIP	Clean in place
DO	Dissolved oxygen
DOE	Design of experiments
DW	Dry weight
ESI	Electrospray ionization
FDA	Fluorescein diacetate
FW	Fresh weight
k_La	The volumetric mass-transfer coefficient
MS	Murashige and Skoog medium
OFAT	One-factor-at-a-time method
OUR	Oxygen uptake rate
PCV	Packed cell volume
SIP	Steam in place
STB	Stirred tank bioreactor
UPLC	Ultra performance liquid chromatography
UPLC-DAD-MS	Ultra-high-performance liquid chromatography–diode array detector–tandem mass spectrometry

.

1. INTRODUCTION

New technologies to produce plant-based foods are needed to fulfill the increasing demand of a growing population with healthy and sustainable food. Present agricultural land use is not sustainable for this increasing demand if dietary habits of consumers stay alike. Agriculture causes the most remarkable amount of global emissions and is a significant burden for the environment (Poore and Nemecek, 2018). Changes are needed to reduce negative environmental effects and to save decreasing water supplies. Also, urbanization drives the food production from the countryside towards build environment. Plant cell cultivation could offer an attractive new method for this rising demand for novel food production. It provides a chance to cultivate healthy plant-based raw material under a closed and controlled environment inside bioreactors (Nordlund et al., 2018).

Plant cells offer several advantages compared to cultivated whole plants. Plant cells can be maintained in a simple media and are relatively easy to grow. Cultivation can be carried out in a closed environment in stable conditions, without negative impacts of pests, microclimate, varying land quality, or diseases. Agrochemicals and pesticides are a concern for the consumer. Neither are needed in bioreactors. Consistency between batches is maintainable and a produced stock is easy to isolate and purify. Good manufacturing practices are also easier to apply than with whole plants in agricultural scale (Hellwig et al., 2004).

When compared to microbial or mammalian cell cultures, plant cells can offer a cheaper and safer production platform, also for more complex molecules and proteins (Santos et al., 2017). Many post-translational modifications can be done by them due to plant cells belong to higher eukaryotes. Plant cells do not carry either human pathogens or generate endotoxins (Hellwig et al., 2004).

Plant cell cultures provide also an attractive production platform for many applications, like recombinant proteins or natural products. Natural products, obtained from plant cells have been exploited as drugs like paclitaxel, natural colorants like anthocyanins and pest control chemicals. Also, the cosmetic industry has used them for several years as a raw material or for fragrance purposes (Ochoa-Villarreal et al., 2016).

Main challenges in using plant cells are the slow growth and low yields compared to commercially more familiar production systems, like microbial cultures. Formation and maintenance of homogeneous and stable plant cell culture strain can be problematic. Scaling up of plant cell suspension cultures is also challenging and there is only a limited

amount of successful commercial examples of this. E.g. plant cell's ability to form clusters can complicate cultivation of cells at industrially relevant scale. Still, microbial and mammalian cells have been used in industrial platforms more than 30 years. Versus to this, plant cell technologies have been used for commercial purposes less than 10 years, and they have evolved impressively (Santos et al., 2016).

Plant cells are not yet widely used in commercial food technology, but there are many potential applications. Most of them are secondary metabolites such as colorants, flavor products, or nutritional supplements, which have high value but are hard to harvest from natural whole plant sources or too complex to manufacture synthetically. These compounds could be produced via plant cells and extracted from them, rather than via complex chemical synthesis for example. (Davies and Deroles, 2014) However, the use of plant cell biomass, as it is, as a food source has not considered in many studies. Now innovative concept of using plant cells as a food has been investigated in VTT Technical Research Centre of Finland. Plant cell biomass of certain species as a whole is proven to have great potential for food purposes (Nordlund et al., 2018). In future plant cells could be a healthy and nutritious ingredient for consumers and food markets. Above the general concept of using plant cells as a food, VTT's target is to make plant cell cultivation possible at home environment. This would increase local and consumer-oriented food production.

To get closer for this VTT's final goal, this thesis aims to create a prototype system of a single-use bioreactor and cultivation bag to grow plant cells for food in non-laboratory environment. The first step is to investigate, the key physical factors affecting biomass formation of lingonberry cell suspension culture. Based on the collected data, I will create hypotheses for single-use cultivation bags. Different prototypes of single-use bags will be tested to confirm the hypotheses. Finally, most promising single-use bag and bioreactor model is going to be chosen for further investigations and building of the working prototype.

The next chapter will cover the theoretical background of plant cell suspension cultures and their cultivation both generally and in bioreactors. Also, the current state of single-use disposable bioreactors is covered to give an overall understanding of available commercial options and their advantages and limitations. Also, the reasons behind why these two technologies are wanted to combine into one home usable unity, are discussed to explain the initial motives. Chapter 3 reports the used materials and methods including the experimental setups in bioreactor cultivations and single-use bag prototypes. All results are presented in chapter 4 and discussed in following chapter 5. Conclusions of this master thesis work and future prospects for home usable bioreactor development from my perspective are presented in final chapter 6.

2. THEORETICAL BACKGROUND

Undifferentiated plant calluses can be cultivated in suspension media and propagated under a controlled and aseptic environment in theory to infinity (Xu and Zhang, 2014). This makes utilization of suspension cultures for bioproduction applications really interesting opportunity.

2.1 Plant cell products

After discovering the idea of cellular totipotency by Haberlandt (Krikorian and Berquam, 1969) the whole concept of plant biotechnology and plant cell cultivation have been evolved tremendously. Many kinds of plant cell cultivations, and their applications have been studied. When plant cell cultivations are considered from the industrial point of view, the main interest has focused on high-value natural products. Complex chemical structure of these natural compounds makes them hard to manufacture in synthetic methods. Also, harvesting of plants in interest for the extraction process is usually environmentally unsustainable. These factors have limited the use of these interesting natural products. Plant cell cultivation process can offer an alternative method for sustainable production (Wilson and Roberts, 2012).

The high-value natural products, which plants have evolved e.g. to protect themselves against pests and pathogens, can be used as drugs, disease, and parasite management chemicals, cosmetics and as an aroma product. Natural products can be produced via several methods, depending on their complexity. Plant cell cultivation offers a powerful production system, especially for complex natural products. (Ochoa-Villarreal et al., 2016) Plant-based natural products, also known as secondary metabolites, have been used for example in commercial pharmaceutical production for several decades. Good example of this is *Phyton Biotech* (www.phytonbiotech.com), which produces sustainable anticancer secondary metabolite called paclitaxel using plant cell suspension culture with a scale of 75 000 L (Huang and McDonald, 2009).

Nowadays, also interest towards commercial production of heterologous protein products via plant cell cultures has increased (Wilson and Roberts, 2012). *Protalix Biotherapeutics* (http://protalix.com/) produces a recombinant human glucocerebrosidase GCD, taliglucerase alfa via transgenic carrot cell suspension cultures in ProCellEx™ platform with disposable bioreactors (Tekoah et al., 2015).

From the point of view of food production plant cell cultivations have been used mainly for the production of food additives. EU-founded project NUTRA-snack aimed for launching safer and healthier ready-eat food and snacks supplemented with natural antioxidant/radical-scavenging compounds extracted from *in vitro* plant cell cultivations (Rea

et al., 2011). Phytochemicals extracted from plant cell cultivations could be for example commercially used is food products as an ingredient of pigments, like anthocyanins and betalains (Nosov, 2012). Concerns and consumer demands about synthetic food colorants have increased the interests of natural alternatives (Oplatowska-Stachowiak and Elliott, 2017). Appelhagen *et al.* studied the effective production of anthocyanin in transgenic tobacco cell suspension culture and reached very high production yields. Still, for further applications in a field of food products, they believe that some other plant species should be used as a host organism (Appelhagen et al., 2018).

Aromas and flavors cover nearly a quarter of the food additive markets. Plant cell cultures can be used to provide natural flavors like vanilla or spearmint. Consumers demands of sustainability and higher prices of natural products increase the interest towards plant cell-based options for flavor production. There are dozens of flavors, from liquorice to saffron, found from plant cell cultures indicating the big potential of this field (Scragg, 2007).

In addition to research, also some commercial applications for plant cell-based food ingredients have still occurred in past years. For example, former Diana Plant Sciences (USA) launched in 2013 nutraceutical cocoa powder made via plant cell technology in bioreactors, but the product is not in markets anymore (NUTRA, 2013). Today, for example, Diana Nova, part of the nutrition segment of Symrise (Germany) is working with plant cell cultures to produce actives for food, health and cosmetic industry (Symrise, 2018). Active Botanicals Research also got Novel Food authorization from EU Regulation to use their plant cell based Acetos 10P (*Lippia citriodora*), Teuopol 10 P and 50 P (*Ajuga reptans*) and Echinan 4P (*Echinacea angustifolia*) as food supplement ingredients. Now final product development can be started for them, used as independent food supplements or with other functional ingredients (Fremont, 2017).

Despite successful research and improvement of culture and cell lines and production technology, most of the plant cell-based applications have not reached the commercial success mainly due to the high costs of the production in relation to the profitability of the final product (Davies and Deroles, 2014). Also, stability issues in the long term and varying product yields complicate the commercial success of plant cell culture-based products (Appelhagen et al., 2018).

Many plant-based secondary metabolites provide health beneficial properties. For example many polyphenolics, like anthocyanin mentioned earlier, are broadly available in many fruits and vegetables. They have many biological benefits, like anticancer and anti-inflammatory activity as for prevention against cardiovascular disease and obesity (Li et al., 2017). Studies highlight also environmental importance of plant-based dietary. For example, Poore *et al.* published an extensive article about environmental impacts of different major foods through the whole supply chain, showing that vegetable alternatives are superior option (Poore and Nemecek, 2018). Still, limitations for agricultural land

use and for example challenges due to global warming and reduced water supplies (Alexandratos and Bruinsma, 2012) drive development of new plant based-food production technologies. For example, avocado's global average water footprint is near 2000 L/kg (Mekonnen and Hoekstra, 2010). Also, urbanization and centralization of the population to big cities enforce enabling the food production effectively not only in countryside but also where most of the consumers are, in a build environment.

New technologies are occurring to markets constantly to answer to the rising demand for more sustainable and local food production. Consumers' willingness to be part of the food production chain has increased steadily and interest towards local or even self-produced food are rising (Newman and Nixon, 2014). New farming methods like vertical and hydroponic farming or novel food ingredients like insects are becoming more general. Applications, where consumers grow their food ingredient on their own, are also getting more common. For example Helsieni is offering a growing kit for oyster mushroom production (Helsieni, 2018). For cellular agriculture applications, homegrown algae are probably the most common example and e.g the company called AlgaeLab is offering kits, parts, and supplies for Spirulina cultivation under the home environment (AlgaeLab, 2018). Based on this rising interest towards novel food solutions, it seems that consumers would be open also for production of plant cells in the home environment (Räty, 2017). Plant cell cultivations offer great opportunity to produce food in the closed, controlled and aseptic environment, without the concern of weather or seasonal. Home growing also solves concerns about reduced biodiversity or ethical problems related to rare plant species resulted because of e.g. global warming.

Possibilities of using plant cells as a food are not studied widely. That is mainly because the focus has previously been in production of the high-value products derived from plant cell cultivations, not in a created biomass itself. Nordlund *et al.* (2018) showed, that plant cell cultures had great potential for food purposes. The study evaluated the nutritional quality of three berry species, cloudberry, stoneberry, and lingonberry and provided basic data about the suitability aspects. Results showed that plant cells have a high protein content, 13.7-18.9 %, with a high amount of important amino acids. The cell mass is rich with fiber, 21.2-36.7 %, and unsaturated fatty acids. These results indicate that cultivated berry cells have even better nutritional quality than natural berries. The taste of fresh cell mass is mild and pleasant and reminds of natural berries, but is less intense. Structure and mouth feel of the cells is bit sandy and coarse. With freeze-drying, the cell mass can achieve a structure which melts into a mouth (Nordlund et al., 2018).

Eibl *et al.* (2018) described recently also use of *Theobroma cacao* cell suspension culture for chocolate production. *T. cacao* cells were cultivated over 16 days in wave bioreactor with 6 L working volume and finally around 50 g/L fresh cell mass was harvested. Cells were freeze dried and used for production of 70 % dark chocolate. Taste was described as intense and complex, having strong citrus and berry aroma (Eibl et al., 2018).

At the moment many processing methods are studied to create new innovative technologies to provide natural and pleasant food products from plant cells with nice taste, mouth feeling, and with visual appearance. This can involve for example enzymatic or mechanical processing steps. Still, before plant cells can be considered as a food product, a long path with novel food regulation must be first walked through. This requires more detailed studies about the production, and detailed composition and safety assessments to prevent possible allergenic or toxic effects (Nordlund et al., 2018).

2.2 Plant cell suspension cultures

Plant cell suspension cultures consist normally from undifferentiated clusters of a plant, also called calli. These are propagated in a liquid media to create stable suspension culture, where cells grow either individually or in aggregates. Available nutrients of liquid medium are ideally exploitable through the whole cell surface (Shaikh et al., 2018). As mentioned earlier, plant cell suspension culture offers a platform which is independent of the variation of weather conditions, pests, and risk of gene flow into the environment (Xu and Zhang, 2014). Culture conditions are sterile and well-controlled: the consistency of growth circumstances can be maintained from batch to batch. Plant cell cultures combine plant capacity to produce complex compounds to the benefits of contained and controlled microbial cultivations (Xu et al., 2012). Still, even though external circumstances can be easily maintained, suspension cultures can face stability problems. Plant cell suspension cultures can be genetically unstable and undergo unplanned variations leading to unstable secondary metabolic yields and heterogeneous cell populations (Chattopadhyay et al., 2002).

Same basic principles apply to the cultivation of all plant, microbial or animal cells, but there are also some differences (Hellwig et al., 2004). Some of which are presented in a table 1. below. These and other typical features will be introduced in more detail in the following sections and they should be taken into account when considering the scalability of plant cell suspension cultures.

Table 1. *Common features of plant and microbial cell cultures, adapted from (Oksman-Caldentey and Barz, 2002).*

Feature	Plant cell culture	Microbial culture
Cell size	Big, diameter of 20-200 µm	Small, diameter of 1-10 µm
Doubling time	Days	Hours
Growth rate	Slow	Rapid
Aggregate formation	High	Low
Shear sensitivity	Moderate, high to low	Low
Oxygen demand	Low	High
Stability	Unstable	Stable
Foaming	Sometimes occurring	Often high
Inoculum density	High	Low
Cultivation time	Weeks	Days

2.2.1 Features and growth of plant cells

The growth of the plant cells can be divided into three main types depending on their carbon and energy source: hetero-, photomixo- and photoautotrophic. Autotrophic plant organisms use inorganic carbon for biomass formation whereas heterotrophic organisms rely on organic carbon source. From the energy source point of view, phototrophic cells utilize light energy while heterotrophic cells receive their energy from oxidative reactions of organic substances. Mixotrophic organisms instead can utilize various carbon and energy sources. To be able to use several carbon sources, the mixotrophic organism needs different biochemical machinery for autotrophic than heterotrophic growth, but the amount of photosynthetic machinery can be reduced when organic carbon like sugars are available (Wilken et al., 2014). Normally undifferentiated plant cells use sucrose or glucose as their main energy source.

Growth of plant cell suspension cultures follows the same model as microbial cultures. First cells induce cell division and adapt to new cultivation environment during a lag phase, followed by period of active cell division. The cycle ends to a period where cell division slows down, eventually leading to cell lysis. Growth curve normally forms an S-shaped curve, including lag, exponential, linear and decreasing growth and stationary phases leading finally to the death of culture, like with microbial cells (George et al., 2008a).

The size of a single plant cell is normally 20-200 µm in a diameter, which can be 10 to 100 times more compared to a bacterial cell. The doubling time of plant cells is relatively long (20-100 h), depending on the species, compared to bacteria (0.5-1 h), yeast (2-3 h) or even to mammalian cells (24-48 h). Some plant species, for example tobacco with doubling time of 12 h, grow faster. (Huang and McDonald, 2009). Especially tobacco cell line Bright Yellow 2 (BY-2) is popular due to its effective growth rate for plant cells (Hellwig et al., 2004).

Plant cells tend to form aggregates and big cell clusters in suspension cultures. This is caused by the failure of cells to diverge from each other after cell division and also due to the increased cell adhesion caused by the extracellular polysaccharides secreted by cells especially at the later stages of the growth. The size and shape of cell aggregate vary depending on the species, inoculum strategy, stage of the cultivation, culture conditions, used media, and bioreactor type. The aggregation can be beneficial in a sense of promoting the cellular organization and differentiation improving the important secondary metabolite production (Huang and McDonald, 2009). For example correlations between cells aggregate size and anthocyanin production have been observed with some species. In strawberry suspension culture, production of anthocyanin was higher in cell clusters with an average diameter of ~390 µm than smaller ones with ~200 µm diameter (Edahiro and Seki, 2006). Experiments with ohelo cells (*Vaccinium pahalae*) verified the same observation. Ohelo belongs to the same genus with lingonberry, which are studied in this work.

Cell aggregates with a diameter of less than 500 µm accumulated most of the pigment in experimental cultures (Meyer et al., 2002). Generally, the formation of big cell aggregates is seen as a negative thing. Big clusters can prevent the mass transfer of nutrients and oxygen, light and other microenvironmental factors to the inner cells of the aggregate and in this way reduce the cell growth. Big aggregates are also problematic in bioreactor operation. They expose cells to hydrodynamic stress which can cause damage to the cells on the surface of the aggregate (Huang and McDonald, 2009).

Morphology of plant cells depends on the species, media composition, and stage of culture. Some cells, for example tobacco, try to reach the transition to spherical to elongated shape after cell division. They tend to form unbranched chains of sausage-shaped cells (Su, 2006). Both formation of cell aggregates and the morphology of the culture, as well as the concentration, stage, and conditions of the culture affect the rheology of the suspension. Elongated and filamentous cell clusters can tangle together and form a cellular network. This can induce decreased packed cell volume (PCV) and noticeable viscosity of the suspension broth (Huang and McDonald, 2009). Changes in cell morphology and culture broth may result in non-Newtonian behavior of the suspension (Werner et al., 2014). To sum: the formation of aggregates and rising cell density leads to an increase in viscosity.

2.2.2 Oxygen demand

For growth and aerobic metabolism cells need oxygen, which is the most important gaseous substrate of the media. Oxygen uptake rate (OUR) indicates the oxygen demand of the culture. OUR varies normally between 5 to 10 mmol-O_2/L/h with different plant cells. The demand for oxygen is relatively low, due to the slow metabolism and growth, compared to microbes which have OUR value typically between 10 and 90 mmol-O_2/L/h. Even though the demand for oxygen is relatively low, the high cell density and viscous cell suspension create challenges for maintaining the dissolved oxygen (DO) of the media and the oxygen mass transfer on a suitable level. To achieve the needed OUR in a bioreactor operation, the volumetric oxygen mass transfer coefficient (k_La) is typically between 10 and 50 h^{-1}. With microbial cultures, the k_La value ranges normally from 100 to 1000 h^{-1}, which is clearly higher compared to plant cells (Huang and McDonald, 2009).

2.2.3 Shear stress sensitivity

Most plant cells are believed to be easily harmed by shear stress due to their big size, rigid cellulose polysaccharide matrix cell wall, and their tendency to accumulate water in vacuoles. Still, the sensitivity varies a lot depending on the species and stage of growth. E.g. tobacco cells are very resistant to shear stress. (Su, 2006). Normally cells start to soak up even more water at the end of exponential growth and at the beginning of the stationary phase. Huge vacuoles can fill up to 95 % of the cell volume. This exposes them to even

more shear sensitivity (Huang and McDonald, 2009). Physiological parameters like viability, respiratory activity, changes in metabolism, extraction of the intracellular compounds like proteins or metabolites and changes in morphology can be used to investigate the effect of shear stress. The amount of caused damage is linked with the intensity and length of the exposure (Su, 2006). The higher the viscosity of the broth, the higher power input is needed to maintain the same cultivation conditions. Too high power input may cause damage to sensitive plant cells (Werner et al., 2014). Still, the mechanism of hydrodynamic shear stress cell damage is not fully understood because the differences in cell lines, cell wall structure, cell morphology, and aggregate size distribution create so diverse and challenging combination of causality (Huang and McDonald, 2009).

2.2.4 Foaming and wall growth

Plant cells excrete extracellular compounds during the exponential growth phase and, because of possible cell lysis, in the stationary phase. This can lead to foaming of the culture. The extracellular compounds are typically polysaccharides and fatty acids. Medium composition, like high initial sugar concentration, may also cause foaming, as well as aeration strategy. Foaming is a typical problem especially in cultivations which are aerated with bubbles. Too high aeration rates can result growth inhibition also, due to gas stripping of carbon dioxide (CO_2) and ethylene (C_2H_4) and formation of disadvantageous gases or other volatile metabolites (Huang and McDonald, 2009). Formation of the foam layer is problematic because it can capture cells and prevent the supply of nutrients and oxygen to them. This leads to lower biomass formation and yield losses (Abdullah et al., 2000). Secondary metabolites and proteases extracted by stressed cells can also induce the formation of the thick and sticky layer to walls of cultivation vessel and on impeller shaft, sensors and sampling port. This phenomenon is called wall growth and it can disturb desirable flow patterns, complicate the operation of a bioreactor, prevent sampling and also decrease the yield by entrapping cells (Abdullah et al., 2000; Huang and McDonald, 2009).

2.2.5 Temperature and pH

The optimal pH and temperature depends on the used species. Normally, cultivation temperature varies between 17 and 28 C^o. Cultivation temperature can also affect to the rate of produced secondary metabolites. The pH of the growth medium is normally adjusted before autoclaving between 5 and 6. Autoclaving of the media, which includes sucrose, lowers the pH value slightly (Werner et al., 2014). Pre-autoclaving pH value affects also to the partial hydrolysis of sucrose to glucose and fructose during autoclaving. This can have an impact to growth (George et al., 2008b). Level of culture pH is not normally adjusted during growth like with microbial cultivations. Plant cells adjust the pH on their own to suitable level depending on the stage of growth. This is caused by the uptake of nutrient cations and anions, and causes the variation to the number of hydrogen ions and

thus to pH level during cell growth. During ammonia assimilation, pH starts to decrease. During nitrogen uptake, pH increases (Ramachandra Rao and Ravishankar, 2002). Nitrogen uptake and the decrease of pH is normally most notable within the first couple of days. During stationary phase pH decrease might also be caused by ammonium release of dead cells (Werner et al., 2014).

2.2.6 Secondary metabolite formation

The production of secondary metabolites is normally strongly linked to the cell's stage of differentiation. As mentioned earlier, cultured plant cells are considered undifferentiated. With these cells metabolic pathways for secondary metabolites are normally not active. In most cases, the maximal biomass formation is achieved when cells are maintained in a low differentiation stage. If the aim is to produce as much as possible biomass with good secondary metabolite yield, the balance between both production rates must be maintained. Heterogeneous cell composition can make that really challenging (Deroles, 2008). For example in a case of anthocyanin, normally fast growing cells lose their ability to produce this wanted secondary metabolite. These cells have shorter doubling times and, over extended period of cultivation, take over the cells which are producing anthocyanin but grow slower (Appelhagen et al., 2018).

2.3 Plant cell cultivation in bioreactors

Cultivation of plant cell suspension can be carried out in shake flasks for small-scale experiments. The production capacity and also the possibilities of monitoring the growth are limited in the shake flask scale. When interests are towards industrially relevant production capacity, cultivations can be scaled up in different types of bioreactors. Bioreactor system offer a sterile and well-controlled environment for cultivation. First large-scale plant cell cultivations were carried out already in 1959 with tobacco cells. In many cases, the same bioreactors which are suitable for microbial fermentations can be applied also to plant cell cultivations, with some modifications. Most common type of bioreactor for plant cell cultures is stirred tank bioreactor, which is widely used in industry. Many other available models e.g. airlift, rotating drum and packed bed bioreactors can be also used for plant cell cultivations (Oksman-Caldentey and Barz, 2002).

Even though plant cells can be produced in industrial scale by using bioreactors, scaling up can bring up many challenges. Typical features of plant cell suspension cultures, discussed in the previous sections, should be taken into account when choosing the bioreactor type or process conditions. In principle, optimization of physical processing conditions can make a remarkable difference for production yields. Different methods for this are discussed in the next section.

2.3.1 Process optimization to increase the growth of plant cells

There are several strategies to improve culture conditions. Roughly they can be divided into molecular and process development approaches. To maximize the utility, both approaches are required. Especially when plant cell cultivations are used as a source of recombinant proteins, molecular methods like transcription enhancement, translation efficiency improvement, and minimizing post-translational degradation are important (Xu and Zhang, 2014). Of course, these affect not only the production of metabolites but also the growth of the cells themselves.

In this work, I will concentrate only on the physical process development approaches. Optimization of culture conditions e.g. aeration and agitation rate, pH, light intensity, and temperature can increase the yield of culture remarkably. Also, the operation mode should be always considered based on the application. Long lag phase, limiting factor of exhausting key nutrients and the production of inhibiting compounds can prevent batch culture to reach the desired productivity. Other operation models like fed-batch, continuous or perfusion culture can be more suitable for some applications.

Normally process and cultivation optimizations are made by using a one-factor-at-a-time (OFAT) approach. This means that only one parameter at a time is changed when others are maintained constant. Limited range of options can be analyzed and most often the truly optimal conditions cannot be found by the OFAT method. Still, significant yield improvements can be achieved by testing individual parameters through trial and error. The method is regarded as a simple and convenient one, but it does not take into account the several interactions between different parameters. The interactions can have hard to predict overall effects on the outcome of the cultivation (Schmitz et al., 2016). A good example of this is the oxygen uptake (Vasilev et al., 2013). Not only the amount of aeration but also the mixing, shape of the cultivation vessel, filling level, and air pressure can have multiple complex interactions, which all effect to the productivity.

Another strategy for optimizations is the statistical experimental design, also called as a design of experiment (DOE) (Schmitz et al., 2016). With this approach, several parameters can be varied simultaneously and interactions between different variables can be examined. DOE is used in culture medium optimization, but can also be applied in the optimization of physical cultivation factors (Rasche et al., 2016). Based on a statistical model, proposals of optimal conditions can be made even though these exact combinations of cultivation parameters would not have been tested (Schmitz et al., 2016). Still, also DOE needs wide experimental setups and sample amount to create reliable statistical model. Rasche *et al.* (2016) used DOE for optimizing biomass production of pear cell suspension cultures by changing temperature, incubation time, inoculum density and light. After optimization, the biomass yield was almost 2.8 times higher (Rasche et al., 2016).

2.3.2 Aeration and dissolved oxygen

Aeration takes care of three main functions in a plant cell suspension cultures. It maintains the aerobic environment, desorbs volatile compounds, and removes the heat created by the cell metabolism (Murthy et al., 2014). The gaseous composition in suspension cultures is a relevant element influencing to the plant cell physiology. Particularly attention should be paid for O_2, CO_2, and C_2H_4 (Jeong et al., 2006). Lingonberry cells need oxygen for their metabolism and growth but also for secondary metabolite production. In flask cultivations, the gas exchange occurs passively through the mouth of the flask, covered normally by a thin foil. For bioreactor cultivations, gas exchange can be taken place through spargers or by bubble-free aeration through the headspace of the cultivation vessel. Insufficient supply of oxygen can lead to limitations in growth or eventually to death of the culture. However, too strong sparging may lead to unwanted foaming (Gao and Lee, 1992), mechanical shear stress and high power consumption (Jeong et al., 2006). Too high oxygen levels can be also toxic for cells (Chattopadhyay et al., 2002). Unwanted stripping of important growth regulators such as ethylene and carbon dioxide may also occur (Biddington, 1992). Carbon dioxide is in many cases very important nutrient for plant cells and has a positive effect on cell growth and production of secondary metabolites (Chattopadhyay et al., 2002).

The transfer of the oxygen from the aeration source to the liquid media plays a remarkable role in bioprocesses. Dissolved oxygen concentrations between 15 to 20 % air saturations are normally recommended for plant cell cultivations (Kieran et al., 1997). The critical level of DO only for cell growth may be even notably lower than the level cells need for metabolite production (Schlatmann et al., 1995). In general, the need of oxygenation is remarkably lower than with microbial cultures (Ducos and Pareilleux, 1986) but high cell density and viscosity of the broth can reduce the efficiency of oxygen transfer (Han and Zhong, 2003). Still, if the level of dissolved oxygen decreases under critical level direct effect to cell metabolic activity can occur due reduced energy levels. This leads to growth limitations and decreased yields. DO is a critical factor for bioreactor and scale up design to ensure sufficient oxygen transfer (Jeong et al., 2006). For comparison, DO-levels used in different plant cell bioreactor cultivations are collected to a table 2. below.

Table 2. *Culture condition comparison of different plant cell cultivations in a stirred tank bioreactor.*

Plant specie	WV (L)	DO (%)	Flow rate (vvm)	Agitation (rpm)	Tip-speed (m/s)	Reference
Ohelo *Vaccinium pahalae*	10-11	50	0.06-0.07	75- 225	-	(Meyer et al., 2002)
Cloudberry *Rubus chamaemorus*	2	20	0.25	75-275	0.25-0.9	(Nohynek et al., 2014)
BY-2 *Nicotiana tabacum L.*	600	30	0.33	120	2.3	(Reuter et al., 2014)
Neem *Azardirachta indica*	2	30	-	400 and 800	0.94 and 1.88	(Villegas-Velásquez et al., 2017)

2.3.3 Agitation

The amount of dissolved oxygen is strongly related to aeration rate but also to mixing of the cultivation broth. The intensity of mixing, air bubble dispersion, hydrodynamic stress and viscosity define the total aeration of the culture. Plant cell cultures have more complex rheology leading to more complex mixing requirements, compared to bacterial and yeast cultivations (Doran, 1999). The relatively high shear sensitivity of plant cells should always be taken into account when right mixing conditions are considered. On the other hand, the tendency to aggregate leads to sedimentation of cell mass when agitation is not sufficient (Chattopadhyay et al., 2002). Optimal agitation is critical in order to sufficient disperse of the gasses and nutrients without causing damage to cells (Sajc et al., 2000). Especially mechanical agitation may easily damage fragile cells. In stirred tank reactors, low agitation levels with high aeration have been used to create effective mixing in as sensitive way as possible. Air-lift reactors can achieve better oxygen transfer compared to stirred tank reactors, but bubble bursting can create high shear stress. Also bubble-free aeration is an effective and gentle way to transfer gasses to liquid broth without shear stress (Chattopadhyay et al., 2002).

Mechanical stirring can also maintain a smaller aggregate size of cell culture compared to more gentle mixing methods. Still, it seems that aggregate size distribution cannot be controlled by mechanical stirring. Meyer *et al.* investigated the influence of agitation level in stirred tank bioreactor to for aggregate size of ohelo cells. Levels 75, 150, and 225 rpm were used, and results revealed that variation in size distribution of aggregates was not significant despite different agitation speeds. Still, when the highest agitation speeds, media browning was observed, indicating about phenol leaching from lysed cells (Meyer et al., 2002).

In stirred tank bioreactor cultivations the hydrodynamic conditions are mostly related to the impeller design and power input. Many impellers have been developed to create more sensitive but still effective mixing with low agitation speed for plant cell cultivations compared to most common impeller type, Rushton turbine. In addition to the type of impeller, impeller off-bottom distance, the ratio of liquid height to tank diameter and the position and number of impellers and their width affect to the outcome of mixing. With Rushton turbines, mixing creates effective gas dispersal by radial flow pattern. If too high aeration levels are combined with low mixing speed, the impeller may flood. This means that the impeller exceeds its gas handling capacity and flow pattern is changed to incorrect one, mainly buoyant gas-liquid flow (Doran, 1999). Normally moderate aeration and agitation levels can be used for plant cells in stirred tank cultivations, if the viscosity of culture broth does not limit the oxygen transfer.

For example in stirred tank cultivation of cloudberry Nohynek *et al.* (2014) used agitation speeds between 75-275 rpm (0.25-0.8 m/s impeller tip speed) in 5L stirred tank bioreactor (Table 2) with normal Rushton impellers. With all these agitation values the cell viability of culture was maintained between 80-90 %. This indicates that there was not too much harmful shear stress. With low aeration rates, also foaming did not reach the exhaust gas outlet and did not cause any remarkable harm (Nohynek et al., 2014). Even higher agitation speeds have been used with plant cell cultivations. Villegas-Velásquez *et al.* (2017) cultivated *Azardirachta indica* cells in 3 L stirred tank bioreactor with agitation speeds of 400 and 800 rpm. With 400 rpm (0.94 m/s) mixing the cell viability remained on an intermediate level, but with 800 rpm (1.88 m/s) harmful effects were observed and viability decreased (Villegas-Velásquez et al., 2017). Suitable mixing rates need to be estimated case by case based on the used cell line. This increases the challenges related to scaling up of new plant species or cell line.

2.3.4 Inoculum density and age

The rate of biomass accumulation in plant cell cultures can be determined by the density of inoculum (Lee and Shuler, 2000). Generally, fast growing cell lines need diluted inoculum compared to slow growing ones. Also the age and therefore the growth stage of used inoculum can have a severe effect on the growth (Sen and Swaminathan, 2004). In a case of tobacco cultures, increasing inoculum density from 5% to 8% led to a shorter lag phase and faster growth. Because of faster biomass accumulation also limitations in dissolved oxygen are reached earlier with higher inoculum (Raven et al., 2011). For ginseng cell suspension culture the inoculum density of 100 g/L was founded to be optimal for biomass accumulation when four different starting inoculums were examined (40, 60, 80, and 100 g/L), but when the amount of wanted secondary metabolites were followed, 60 g/L inoculum was more suitable (Thanh et al., 2014).

2.3.5 Light conditions

Light can be an important factor for biomass production yield and also a key elicitor for biosynthesis of secondary metabolites (Fazal et al., 2016). For *M. malabathricum* cultures the use of moderate light intensity (300-900 lux) led to highest biomass yield and was also more suitable for pigment production. Very high light intensity above 900 lux limited the growth and were also harmful for pigment production. Also, the light period affected the pigment production. The highest pigment amounts were achieved after continuous light irradiance (Chan et al., 2010).

Formation of blue, purple and red colors in berries is mainly caused by water-soluble vacuolar pigment flavonoid called anthocyanin (Lee and Finn, 2012; Li et al., 2011). Induction of anthocyanin biosynthesis in plant cells can be a response to several environmental changes, and one of those mechanisms is photoinduction (Chalker-Scott, 1999). For strawberry suspension cell culture, increasing the light irradiation intensity from 1.5 to 8.0 klux did not affect to the cell growth, but increased anthocyanin production remarkably from 0.04 to 0.19 mg/g (Sato et al., 1996). Also Kurata *et al.* came to the same conclusion with strawberry cell suspension culture (Kurata et al., 2000). On the other hand, some *Aralia cordata* cell lines can maintain the high anthocyanin productivity levels without any light (Kobayashi et al., 1993). Meyer *et al.* investigated ohelo (*Vaccinium pahalae)* cells, and with these relatives of lingonberry, amount of irradiance did not have a remarkable effect to biomass formation in shake flasks for cells which were pre-cultured under darkness and had no pigmentation at the beginning of the experiment. However, irradiance had a significant influence on anthocyanin production. With the highest irradiance level, anthocyanin accumulation was 125 % higher than with lower irradiance levels. When ohelo cells were cultivated in stirred tank bioreactor with 10 L wv, anthocyanin content started to decrease after time due insufficient light irradiance, 100 µmol m^{-2} s^{-1} at the inner surface of the glass vessel covering 240º of the outer surface of the reactor. When light source was changed and amount of irradiance radiation dose was increased to 240 µmol m^{-2} s^{-1}, anthocyanin production grew notably (Meyer et al., 2002).

The light-dark rhythm can also affect to the secondary metabolite production (Murthy et al., 2014). Light-induced production of secondary metabolites requires the photosensory and photoregulation system to control the expression of responsible genes. Normally photoreceptors are either reversible or irreversible switches depending on whether they can be turned on and off anytime. Light-dark cycle can be provided either in second-scale by using fast light pulses, or by using longer periods of light and darkness in an hour scale. Anthocyanin production for strawberry suspension culture was enhanced by using a 20 second-scale light-dark cycle with 0.75 ratio (15 seconds in light, 5 in darkness). Under an hour scale, continuous irradiation provided the most suitable conditions for anthocyanin production. Neither second nor hour scale light-dark rhythm did affected to the total amount of biomass, only to the portion of produced anthocyanin (Kurata et al., 2000).

When plant cell suspension cultures are scaled up from incubation shakers, the efficient illumination has to be ensured. Constant access to light source is necessary for many plant cell cultures and during scaling up to a stirred tank and wave reactors this can be limited (Schmitz et al., 2016). Level of light energy per unit volume is challenging to maintain in large cultivation vessels. That is why commercial applications might want to concentrate on plant cell cultures which can be cultured under dark conditions. Insufficient amount of diffused light in industrially relevant scale of closed photobioreactors is also challenge in algae production. Algae biomass can be produced for biofuel purposes as a low value product, like plant cells in food purposes. In some bioreactor solutions for algae production, promising technologies for efficient light utilization have been already developed. For example flat plate or horizontal tubular photobioreactors with narrow tube diameter have better characteristics for effective light utilization (Gupta et al., 2015). Innovations from algae production can be utilized also to plant cell production.

2.4 Disposable sigle-use bioreactors

As discussed in previous sections, there are commercial and scientific examples of plant cell cultivations in traditional bioreactor systems. Another strategy for bioreactor cultivation is disposable bioreactor systems. Disposable bioreactors refer to different cultivation platforms, which are meant only for single-use. These flexible production systems can offer several advantages for plant cell cultivations and are interesting especially in a point of view of the home usable bioreactor.

Disposable bioreactors, which are available on markets are normally made from Food and Drug Administration (FDA) -approved sterile polymeric materials like polyethylene, polystyrene, or ethylene vinyl acetate. The complexity of single-use reactor depends on the scale of cultivations. In the simplest situations, single-use bioreactors contain only a sterile container for cultivation and optimal cultivation conditions are created with external devices. These kinds of applications are normally used only with a small-scale cultivations. When the culture volume increases over 1 L, integrated units for measurement and control of process parameters are more often used (Eibl et al., 2010). In larger scale systems the flexible cultivation chamber also needs an external support container or a frame to hold the reactor in a wanted shape (Eibl et al., 2011). The cultivation chambers of single-use bioreactors are pre-sterilized, which eliminates the need for cleaning and sterilization at a site (Eibl and Eibl, 2006).

The history of single-use bioreactor development started in the 1960s and can be roughly divided into three sections. All started with simple single-use cultivation devices and shaken plastic bags. The idea was developed towards disposable membrane bioreactors and pneumatically driven and rocking bags from the 1970s until the late 1990s. Nowadays disposable bioreactors are developed towards scalable bioreactors systems to fulfill the demand of flexible production for continuously changing markets. For example, wave-

mixed, stirred and orbitally shaken single-use reactors have arrived in commercial markets (Eibl et al., 2011). Recently ABEC launched larger single-use system with a working volume up to 4000 L ("ABEC Custom Single-Run Scalable Solutions," 2018). The most common types of single-use reactors will be discussed with more details in upcoming sections.

2.4.1 Benefits and limitation of disposable bioreactors

Disposable bioreactors offer several advantages compared to conventional models, like stainless steel stirred tank bioreactors, which are widely used in industry. Disposable solutions increase the production flexibility and enable decentralized manufacturing. Also, the operation of these systems is easy and simple. Disposable bioreactor technology allows the changes in production site by lowering the change over time remarkably: process trains can be assembled quickly from ready to use modules and scaling up of each process is more flexible compared to traditional hard-piping processes (Gottschalk, 2009; Shukla and Gottschalk, 2013). Because of single-use bioreactors are supplied sterile, savings can be achieved through easier cleaning protocols and validation of cleaning process. This also reduces the changeover times between batches when time- consuming disassemble and reassemble process with steaming and cleaning can be eliminated. Utility costs decrease when steaming-in-place (SIP) is not needed, leading to energy savings (Shukla and Gottschalk, 2013). Process equipment related to clean-in-place (CIP) and SIP and their work instructions can be eliminated which increases the savings and working safety. (Brecht, 2009) The main advantage of these sterile single-use production platforms are still the minimal risk of cross-contamination in a multiproduct facility (Gottschalk, 2009). The risk of contamination from process air or steam is also reduced. The transparent material of the reactor vessel also enables the visual evaluation and illumination of culture (Brecht, 2009).

Despite the many advantages of single-use cultivation technologies they face some key challenges. The challenges are mainly related to their performance issues, lack of standardization, and limited scale and the number of available commercial options. Scalability of available commercial single-use bioreactors is mainly from laboratory scale reactors to the maximum working volume of 4000 L. Compared to traditional bioreactor technology the scale of available options is very limited. Primarily reason behind this problem is the lack of strength of the used plastic material. It should be still taken into account, that scalability problem can be partly related to the demand on the market, not only technical restrictions. As far there are still not that many vendors available in markets of single-use technologies, this lack of competition may limit the development of available commercial models (Shukla and Gottschalk, 2013). Systems with larger working volumes will probably enter the market in future based on the increasing interest of the customers. Also horizontal scale-up strategy can be used to overcome problems related to the mate-

rial strength (Andrews and Roberts, 2017). For example, Protalix uses ProCellEx™ platform with 400 L disposable vessel cultivating several reactors side by side. Horizontal scaling enables the simpler maintenance of stable culture conditions compared to traditional scaling up approach. When sizes of bioreactors gets larger, normally temperature and nutrient gradients start to occur and disturb cultivation (Yoshida et al., 2017). Also, the lack of process control compared to the traditional methods is limiting the effective use of disposable bioreactor technology. Disposable non-invasive process devises such sensors and valves for online monitoring have not developed yet enough to fill the requirements of markets (Brecht, 2009).

Another challenge related to the plastic materials used in a reactor vessel apart from the strength of it is the possibility of released compounds from it. The absence of quality regulation of used disposable materials and worry about possible leachables and extrables from them slow the adaptation of this new technology. Problem is especially remarkable among biopharmaceutical companies, where the purity requirements and contamination risk of final products are higher. The effect of possible leached compounds from plastic and their effect on cell growth and activity should be considered. These leached compounds are chemicals which migrate in normal process conditions from the contact surface to the media. Extractable compounds instead are released from plastic bioreactor under extreme conditions, such as high temperature or in a presence of a certain solvent (Shukla and Gottschalk, 2013). In future suppliers of disposable reactors need to provide necessary technical data for customers to validate the safety of used equipment and materials. The field needs still more standardized ways for doing this validation. To ensure this, a collaboration between different industry groups has started to create strategies to determine the sufficient validation of quality by the vendors (Mokuolu, 2018).

Disposable bioreactors can be classified based on the power input type causing the agitation into dynamic and static systems. Dynamic systems can be furthermore divided into hydraulically, pneumatically and mechanically driven bioreactors or their hybrid systems. Dynamic systems provide better mass and energy transfer and can be more suitable for higher cell density cultivations than static ones (Eibl et al., 2011). Illustrative chart of disposable bioreactor classification is presented in the Figure 1 below. Most common group of commercially available applications are mechanically driven single-use bioreactors with oscillating movement (Pilarek et al., 2018).

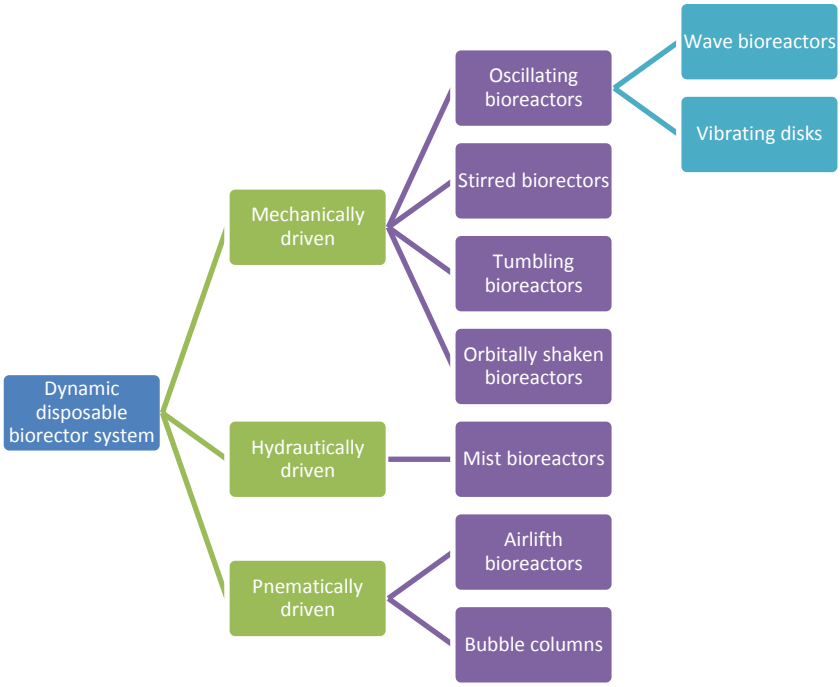

Figure 1. *Classification of different dynamic disposable bioreactor types based on the general energy input categorization. Graph modified from (Lehmann et al., 2014).*

The size and form of the used flexible plastic cultivation bag can also determine the possible agitation mechanism for that type of solution (Pilarek et al., 2018). The most common types of disposable bioreactors are presented in the Figure 2 below. Some of these models and interesting new studies related to disposable bioreactors will be discussed in the upcoming sections below.

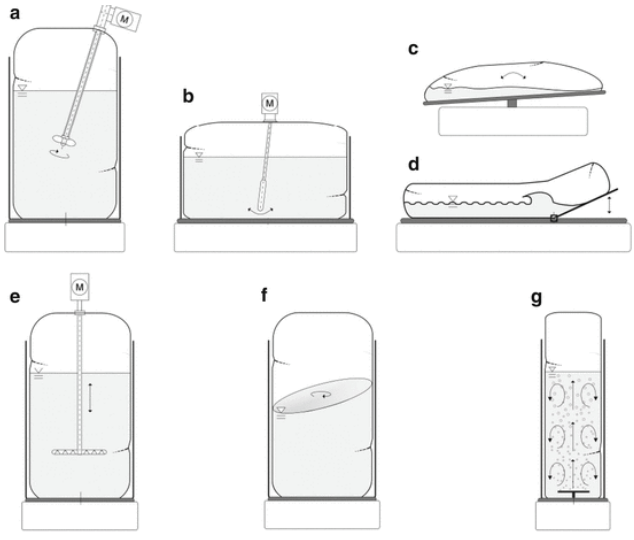

Figure 2. *Diagram of different disposable bioreactor types. (a) Bioreactor with rotating stirrer, (b) Bioreactor with tumbling stirrer, (c) Bioreactor with rocking platform, (d) Bioreactor with raising platform, (e) Bioreactor with vibrating disk(s), (f) Orbitally shaken bioreactor and (g) Bubble column bioreactor. Modified from (Lehmann et al., 2014)*

2.4.2 Wave bioreactor

Wave-bioreactors (Fig. 2 c) consists of disposable and sterile multilayer plastic bag which is partially filled with culture media and cultivated cells with the air inlet and -outlet tubing for the gas exchange. The bag is placed on a rocking platform to create a wave motion into the cultivation media to provide proper mixing and bubble-free surface aeration from the headspace (Andrews and Roberts, 2017). The mass transfer rate can be adjusted by changing the angle and speed of rocking plate and bag filling ratio (Shukla and Gottschalk, 2013). This method provides gentle mixing and risk of shear stress and foam formation is minimal. Because of these advantages it is suitable for plant cell cultivation (Andrews and Roberts, 2017).

Wave-mixed reactors are best suitable for small or medium scale cell cultivations with relatively low oxygen demand, especially for the cultivation of non-Newtonian cultures like tobacco cells (Eibl et al., 2010). Wave-system bioreactors alter from each other by different control mechanism, bag-design, installed sensors and movement type of used platform and they can be operated with either, batch, fed-batch or perfusion mode (Eibl et al., 2011). According to Pilarek *et al.* wave type of bioreactors dominate at this moment the single-use bioreactor market, compared to other available systems (Pilarek et al., 2018). There are two main vendors: GE Healthcare (USA), which offers ReadyToProcess WAVE™ system up to 25 L (GE Healthcare, 2018) and Sartorius AG (Germany), which offers BIOSTAT® RM rocking systems up to 100 L (Sartorius, 2018).

2.4.3 Stirred bag bioreactor

Like traditional stirred tank bioreactors made from stainless steel or glass, stirred bag reactors (Fig. 2 a,b,e) also consists of cultivation vessel supplied with aeration device like microsparger or sparger ring and different types of agitators like e.g. rotating impellers. Cultivation bag can be for example cubical or cylinder shaped, supported by an external steel structure. Gas filters, sampling ports and integrated control mechanisms and sensors can be used depending on the requirements of culture. External heating and cooling jackets can be also used (Eibl et al., 2009). Stirred bag cultivation systems are suitable also for higher product quantities, from medium to high cell densities, because their mixing can be adjusted to a more effective level compared for example to wave bioreactor.

In a case of plant cell cultivation, the risk of high shear stress should be still considered. Generally, these systems are more suitable for applications where shear forces are less important. Mechanical stirring might damage cells or when lower stirring rates are used with high cell density cultures, dissolved oxygen levels can drop under critical level (Doran, 2013).

Another challenge related to single-use stirred bag bioreactors, is the connection point of rotating stirrer and the static part of the bioreactor, creating a possible contamination risk

if operated not under sterile conditions (Junne and Neubauer, 2018). Commercial applications are available from mL up to m^3-scale (Junne and Neubauer, 2018), for example from Merck Millipore (Germany), which offers Mobius® Single-Use Manufacturing platforms from 3 L up to 2000L (Merck Millipore, 2018) and Eppendorf (Germany), offering BioBLU® platform from 100 ml up to 40 L working volume (Eppendorf, 2018).

2.4.4 Pneumatically mixed bioreactor

For pneumatic mixing, several applications have been investigated. Most common types are bubble column (Fig. 2 g) and airlift bioreactors, which are well-known models also among the traditional stainless-steel bioreactors. Mixing is done without impellers, only by bubble motion (Furusaki and Takeda, 2017). Air sparger is normally placed to the bottom of a cylinder or conic cultivation vessel. In an airlift reactor, two coaxial vertical cylinders are placed within each other and sparger is positioned normally under the inner cylinder to create rises section. This design improves the medium circulation pattern and removes dead zones, which tend to form especially into the sides of bubble column vessel. (Khanahmadi and Paek, 2017) The use of bubbles for aeration and mixing of cultivation broth might be harmful to most sensitive plant cells, because of the bubble bursting. Direct aeration may also cause severe problems with foaming and wall growth, although the same problem should be considered also with stirred bioreactors (Eibl and Eibl, 2008). Commercially available options are offered e.g. by Cellexus Ltd (UK) with CellMaker^{TM} providing the working volume of 8 and 50 L (Cellexus, 2018), but this platform is more suitable for microbial cultivations than for plant cells.

2.4.5 Orbitally shaken bioreactor

One promising option to wave and stirred disposable bioreactors are the orbitally shaken bioreactors (Fig. 2 f). They offer bubble-free aeration and homogenous liquid distribution with a simple design. The oxygen transfer to cell suspension is achieved via surface aeration and orbital rotation of the vessel. The rate of transferred oxygen depends on the volumetric power input and the size of the total gas-liquid area. Surface area can be increased by increasing the inner diameter (Klöckner et al., 2014). Typical height over diameter ratio of the orbitally shaken bioreactor is from 1/1 to 1/1.5 and for the ratio between the height of liquid to a diameter from 0.5/1 up to 1/1 (Werner et al., 2010). Aeration rate is also affected by the thin and renewable liquid layer which stays at the wall and bottom of the reactor for a short moment in every circulation. The simplicity of the scaling up is one advantage of orbitally shaken systems. Steady shaking motion is well known and easy to predict and the same agitation principle is used on a laboratory scale cultivations. Either square or cylindrically shaped vessels are normally used. Corners in a square reactor act as baffles creating a more chaotic mixing and increasing the oxygen transfer, but also cause shear stress (Klöckner et al., 2014). Orbitally shaken reactors are considered as a good option providing more homogenous shear stress pattern compared to stirred

bioreactors for example with Rushton impeller and this way lead to higher cell counts. Also, uniform energy input and very low foam formation are benefits of orbitally shaken bioreactors compared to stirred reactors (Werner et al., 2010). Commercially available options are from lab scale up to 200 L solutions. For example, Swiss company Kuhner manufactures orbitally shaken bioreactors from 3 up to 200 liters (Kuhner, 2018). Also prototypes of bigger orbitally shaken applications have published. Zhang *et al.* designed and manufactured platform with shaking diameter of 10 cm and shaking speeds up to 45 rpm for 2000 L cultivations with working volume of 1000 L (Zhang et al., 2009).

Still, when size of orbitally shaken vessel increases, more power is needed to create sufficient mixing pattern into the bioreactor. Scaling up of orbitally shaken bioreactors above certain level is really challenging. This is due to high masses and thus powers needed for maintaining the controlled orbital movement and to hold the vessel still above moving platform.

2.4.6 Multi-chamber bioreactor

One interesting idea for single-use bioreactor design is the multi-chamber bioreactor. The main goal of this invention is to lower the turndown ratio and this way speed up and ease the production. The design showed in Figure 3 below consists of the two-chamber bioreactor, in which the outer chamber encloses the smaller inner chamber and control of both chambers is carried out via one control unit.

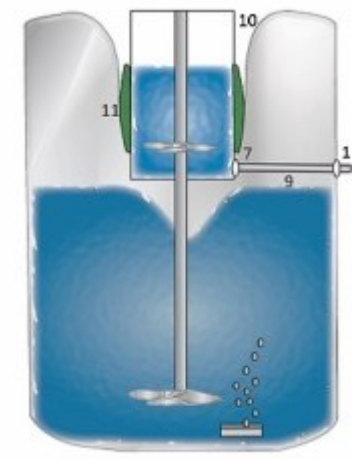

Figure 3. *A schematic diagram for side cross section in the two-chambers assembly (Amer and Ramsey, 2018).*

Chambers are connected with each other via inner tubing system which enables the transfer of culture broth with a peristaltic pump. Bioreactor systems do not need to be opened during the transfer, which lowers the contamination risk and simplifies the operating. In this prototype, mixing was carried out with two impellers. Expansion capacity of culture volume by the system is from 1 L to 50 L. The prototype proved that the concept of

multiple chamber bioreactor reaches the same performance than commercially available bioreactors (Amer and Ramsey, 2018).

2.5 Home bioreactor

From sterile and highly controlled world of disposable bioreactors and e.g. plant cell cultures for the pharmaceutical industry, the jump to sometimes bit messy home kitchen is enormous. How can we take cultures, which are normally handled by laboratory experts under sterile laminar flow or even in clean rooms, to the totally different environment, full of potential contamination sources? The concept of the home usable bioreactor and growing plant cells in the home environment was studied by Nico Räty (2017). He aimed to design and produce prototype bioreactor which would be as easy to use as any other domestic appliance found from home kitchen. Concept called Home Bioreactor was created and a modular prototype of bioreactor design presented in a Figure 4 below was produced (Räty, 2017).

Figure 4. *A prototype of Home Bioreactor design made by Räty, resembles design lamp and is designed to fit next to other kitchen appliances at home-environment (Räty, 2017).*

Working mechanism of the Home bioreactor resembles coffee machine. Reactors contains two sections. One is for single-use cultivation bag including cells and needed medium components and other is a container for adding water. When reactor is switched on, water is pumped to a cultivation bag and cell culture is kept under optimal growth conditions (Räty, 2017).

Even though the concept and design of Home bioreactor was created by Räty, several factors related to the actual cultivation chamber need still further investigation. Plant cell

cultures need to be cultivated under sterile conditions to avoid contamination of the culture. Disposable bioreactors can offer a solution for this dilemma in a home environment. The operation of bioreactor should be as easy as possible without worries of controlling several cultivation parameters. Manufacturing costs of single-use cultivation bag should be as low as possible because the aim is not to produce purified high-value end products like with pigments or pharmaceutically active compounds, but food. All these factors, in addition to optimal growth conditions for chosen cell line should be taken into account when designing the single-use cultivation bag for the Home bioreactor.

3. MATERIALS AND METHODS

3.1 Used strain, medium and culture conditions

For this study tobacco and lingonberry cell suspension cultures were used. The main interest was on the lingonberry strain, but for comparison also well-characterized tobacco cells were used.

Tobacco cell line (*Nicotiana tabacum L.*) Bright Yellow 2 (BY-2), was received from Tokyo University and included in VTT Technical Research Centre of Finland's proprietary culture collection (http://culturecollection.vtt.fi/) with strain number of P-120001. During this study, the cell line was maintained by sub-culturing 500 µl of the culture to fresh medium every 7 ± 3 days. For cultivation 250 ml shake flasks with 50 mL working volume were used.

For all BY-2 cultivations Murashige & Skoog -culture medium (MS) (Murashige and Skoog, 1962) was used with the following composition: 4.39 g/L MS medium -powder (Duchefa Biochemicals, M0221), 30 g/L sucrose, 0.2 g/L of KH_2PO_4, 100 mg/L of myo-inositol and 1 mg/L of thiamine-HCl. The pH of the medium was adjusted to 5.8 by using 0.1 M KOH. Medium was autoclaved at 120 ºC for 20 minutes. More detailed chemical composition of MS medium -powder is presented in the Appendix A.

Used lingonberry (*Vaccinium vitis-ideae*) cell line was obtained from VTT Technical Research Centre of Finland's proprietary culture collection (http://culturecollection.vtt.fi/) strain number of P-120092. Lingonberry cell line originates from Finland. The strain was maintained as callus culture on solid medium by sub-culturing the cells regularly. All the lingonberry cells used in every experiment of this study were maintained by sub-culturing 15 ml of suspension cultures to fresh medium every 10 ± 4 days by using a 250 ml shake flask with 50 mL working volume.

Woody Plant medium (Lloyd and McCown, 1980) was used for all lingonberry cultivations with the following composition: 2.46 g/L of McCown Woody Plant including vitamins -powder (Duchefa Biochemicals, M0220), and 30 g/L of sucrose. Detailed composition of Woody Plant -powder is presented in Appendix B. Medium pH was adjusted to 4.8 (control) or during one experiment (bioreactor batch Lingonberry-7) to 7.0 by using 0.1 M KOH. Medium was autoclaved at 120 ºC for 20 minutes. After sterilization 1.95 mg/L 1-naphthaleneacetic acid (NAA) (α-naphthaleneacetic acid; Sigma, Munich, Germany) and 2.2 mg/L thidiazuron (TDZ) (Thidiazuron; Duchefa Biochemie, The Netherlands) was added.

Culture conditions for BY-2 and lingonberry suspension culture are shown in table 3 below. Cells were cultivated in 250 ml Erlenmeyer flasks with 50 ml working volume covered with thin foil. During cultivation, flasks were incubated in the INFORS HT Multitron Standard shaker (Germany). If culture conditions differ for the control conditions mentioned in table 3, it is explained in the experimental description.

Table 3. *Control culture conditions of BY-2 and Lingonberry cell suspension cultures used in this study.*

	BY-2	Lingonberry
Cultivation media	MS	Woody Plant
Sub-culturing period (d)	7 ± 3	10 ± 4
Cultivation temperature (°C)	28	24
Mixing (rpm)	130	110
Illumination regime (h)	Continuous darkness	16:8
Type of light	-	White fluorescent light
Irradiance (μmol m^{-2} s^{-1})	-	80

3.2 Shake flask experiments

During shake flask experiments, the growth of the cell culture was monitored. For plant cell cultivation, commonly used methods for measuring the biomass accumulation are the determinations of fresh and dry weight. Fresh weight (FW) measurement describes also how much water cells have stored inside themselves. Still, due inaccuracy of fresh weight measurement and to define how much actual biomass is created also a dry weight (DW) of cell culture must be measured. Normally both results are presented in a scale g/L. Also, pH change and conductivity of culture media are good correlators of cell growth stage.

In this study to evaluate the growth of cells, FW and DW were measured from samples at chosen time points. For FW measurements either packed cell volume (PCV) method or vacuum filtration method was used. PCV method is based on separating cells from liquid media via centrifugation. For this 10 ml of each culture were pipetted into preweighed 15 ml falcon tube. Sample-tubes were centrifuged at 20 °C with 4000 rpm for 15 minutes. With BY-2 cells supernatant was poured out and with lingonberry cells pipetted away from the sample. The pH and conductivity were measured from the supernatant. Only growth curve measurements were carried out by using PCV-method. During vacuum filtration method, which was discovered more suitable for lingonberry cells, the culture was filtered using a Bühner filter and filter cloth. The supernatant was collected

for pH and conductivity measurements. Separated cells were collected from the filter cloth and FW or PCV was measured with laboratory balance.

The conductivity of each sample was measured with the Jenway 4510 Conductivity Meter (UK) and pH with Jenway 3510 pH Meter (UK) right after separation. Weighted cells were frozen for freeze drying to determinate the DW of the cells.

3.2.1 Comparison growth curves of tobacco NY-2 and lingonberry cell cultures

Inoculum of BY-2 was made from 7 days old cells. Used inoculum density was 25 g/L. For lingonberry, the inoculum for experiments was made from 10 days old lingonberry cultivations. The target inoculum density was 70 g/L. The fresh cell suspensions were transferred to 250 ml Erlenmeyer flasks with working volume of 50 ml. Flasks were cultivated in control cultivation conditions, mentioned in table 3 above.

3.2.2 Inoculum density experiment

Effect of starting inoculum density to biomass formation under a 16-8 h photoperiod was studied in shake flask experiment. Three different starting cell densities 40, 60, and 80 g/L were used for the experiment. For each measurement point, two parallel samples were measured as described in section 3.2.

3.2.3 Gas exchange experiment

To examine the influence of gas exchange, 14 flasks were started from 7 days old lingonberry wave cultivation with target inoculum density of 80 g/L. Half of the flasks were closed normally with aluminum foil, like with control cultivations. Other half had an approximately 1 cm^2 hole in the cap-foil, covered with wound tape, to create more effective gas exchange into the flask. Experiment flasks were cultivated under same conditions in the shaker than other lingonberry shake flask cultivations over 13 days and after that FW, DW, pH, and conductivity were measured.

3.2.4 Dark cultivation experiment

In a first experiment (Dark a) normally under control light conditions (16:8 h photoperiod) cultivated lingonberry strain was transferred to fully foil covered flasks. Two parallel flasks with 40 g/L starting inoculum density were made. Covered flasks were cultivated otherwise under same conditions in the shaker than other lingonberry shake flask cultivations. After 13 days, FW, DW, pH, and conductivity were measured as described in section 3.2. For growth measurements one cultivation was fully used but from another cultivation only 15 ml was used for experimental measurements.

In a second dark cultivation trial (Dark b), the experiment was started from cells cultivated under darkness over 7 days. Inoculum density was 80 g/L. Flasks were cultivated under continuous darkness otherwise maintaining the control cultivation conditions and growth curve of dark growing lingonberry was determined. The aim was to confirm the interesting results related to biomass accumulation from the experiment Dark a and observations related to color formation.

3.2.5 Two-stage cultivation experiment

In this experiment, flasks were started via same protocol than in experiment Dark b and were cultivated first under continuous darkness either until 7 or 11 days and transferred then to control light conditions: 16-8 h photoperiod under white fluorescent light with an irradiance of 80 µmol m^{-2} s^{-1} for rest of the cultivation. From chosen time points FW, DW, pH, conductivity, and color change of culture were analyzed from two parallel samples.

3.3 Wave bioreactor cultivation of lingonberry cell culture

Lingonberry cells were precultured for stirred tank bioreactor experiments in wave bag bioreactor to simplify the handling and production of biomass for required inoculums. For first wave bioreactor cultivations, cells from flask cultivation were used as an inoculum material. Inoculum density was set to 80 g/L. Working volumes between 750 and 1000 mL were used with fresh medium. As a cultivation bag, 2 L Flexsafe RM basic SC bag from Sartorius was used with a working volume of approximately 750 ml. Culture conditions were operated via BIOSTAT CultiBag RM control system and BIOSTAT CultiBag RM rocker (Santorius, Germany) was used for mixing. Rocking rate was adjusted to 20 r, angle to 8º, temperature to 24ºC, and aeration to 0.3-0.5 lpm. Lighting was adjusted as near as possible to control values used in flask cultivation.

3.4 Stirred tank bioreactor experiments of lingonberry cell culture

Stirred tank cultivations of lingonberry cell suspensions were carried out by using Sartorius Biostat B 2.5 L glass vessel stirred tank bioreactors which were controlled through Sartorius DCU Touch control unit and Sartorius BIOSTAT B-DCU supply towers (Sartorius, Germany). Mechanical stirring was carried out by using two Rushton impellers in the stirrer shaft. Sparger-ring near the bottom of the cultivation vessel was used for aeration. Lighting was modified by using adjustable SL 3500 LED-panels with Light Controller LC100 control unit and power supply and driver from Photon Systems Instruments (Czech Republic). LED-panels were located as close as possible to the sides of the bioreactor vessels. Experimental set up is illustrated in a Figure 5 below.

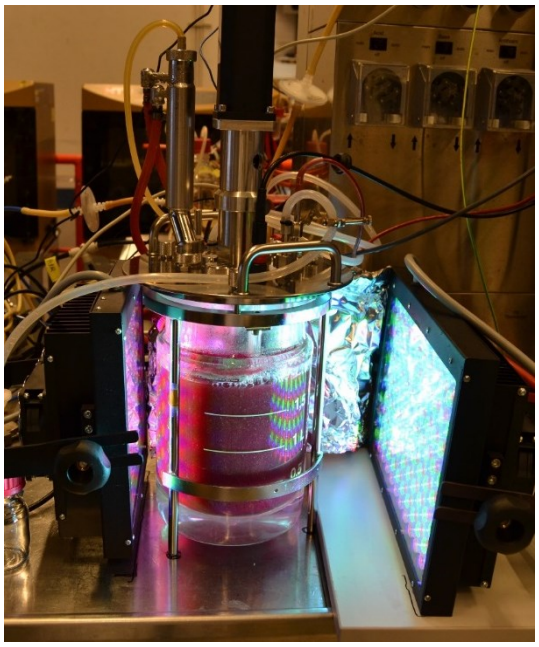

Figure 5. *Stirred tank bioreactor set up with 2 LED panels. During cultivation, the whole system was covered with foil to prevent the interference of other light sources. Picture taken from batch Lingonberry-3 after 12 cultivation days.*

3.4.1 Lighting

Like mentioned above, LED-panels were placed as close as possible to reactor wall. Light conditions were measured with a quantum sensor connected to a radiometer (Li-Cor. Inc. USA) at the inner surface of the reactor facing the LED-panel. Panels provide light from a mixture of blue, green and red LEDs. Through control panel irradiance level and used color of light (red, blue, green or their combinations) were adjusted. When white light was used, a light program which divides target irradiance level equally for each color was chosen from the control panel. Due to the structure of LED-panel consisting of several small LEDs, the level of measured irradiance is varying depending of the location of the sensor in relation to individual LEDs. Exact irradiance values inside a bioreactor cannot be presented because of this.

For runs Lingonberry-1 and Lingonberry-2, one LED-panel per reactor with white light was used. Lighting intensity was adjusted to resemble the lighting conditions used in shake flask and wave cultivations, 60-90 µmol m^{-2} s^{-1}. Same 16-8h photoperiod from shake flask and wave cultivations was also used. Bioreactors were covered with tin foil to prevent the interference from other lights.

For runs, Lingonberry-3 and Lingonberry-4 lighting conditions were modified to increase the amount of light. For run Lingonberry-3 another LED-panel was located to the other side of the vessel to increase the area of lightened cells. The day-night rhythm was removed, and continuous irradiance was used. For both runs Lingonberry-3 and -4, light intensity was increased first near to 200 µmol m^{-2} s^{-1}, and then near 500 mol m^{-2} s^{-1},

finally up to 750 µmol m^{-2} s^{-1}. After 300 cultivation hours amount of light for Lingonberry-4 was increased even more, near 1100 µmol m^{-2} s^{-1} to test the effect of really bright lighting.

During runs Lingonberry-5 and -6 both reactors had 2 LED-panels with continuous irradiance and light intensity was adjusted to level 60 %, correlating with light intensity near 700-800 µmol m^{-2} s^{-1}. For batch Lingonberry-5 light with blue-green color was used and for Lingonberry-6 white light.

For both batches, Lingonberry-7 and -8 similar light conditions were chosen: 2 LED panels per reactors and white light with an intensity of 700-800 µmol m^{-2} s^{-1} and continuous irradiance.

3.4.2 Inoculation of stirred tank bioreactors

The target inoculum density was 60 g/L for every stirred tank bioreactor batch. Inoculums for stirred tank cultivations Lingonberry 1 and -2 were prepared from a mixture of wave and flask cultivated cells. Cells cultivated 7 days in 2 L wave bioreactor with 1 L working volume were used as the main source of inoculum and the amount of cells was topped up with 6 days old shake flask cultivations. For Lingonberry-3 and -4 cells from previous stirred tank cultivation, Lingonberry-2 was used as an inoculum. Lingonberry-5 cultivation was started from Lingonberry-4 cultivations, which was still not red at the end of the cultivation to examine the color formation during growth. Lingonberry-6 was started from red Lingonberry-3 cultivation. Inoculums for batches Lingonberry-7 and -8 were made from Lingonberry-6. Correct amount of cells were weighted and transferred to sterile 2 L bottle with fresh medium under laminar flow.

For runs, from Lingonberry-1 to -4 sterile magnetic stirrers were also used to help inoculation, but for runs -5 to -8, they were decided not to be used to be sure that they do not damage cells. Inoculation bottles were attached to bioreactor under a laminar flow. Cells were pumped from bottles to the reactors by using a peristaltic pump.

3.4.3 Culture conditions

Culture conditions for runs Lingonberry-1 and -2 were set by estimating the possible favorable conditions for lingonberry cells. Stirring and aeration were adjusted as low as possible to avoid shear stress and the rupture of cells. Suitable values were searched for during the first days. The aim of Lingonberry-1 batch was to reach the oxygen limitation, and both aeration and agitation levels were set to low values. Stirring was set to 50 rpm and aeration to 0.1 lpm. For Lingonberry-2 cultivation operation cascade was set to maintain the level of dissolved oxygen first above 20 %. This value was based on the typical conditions used for plant cells in literature but was decreased to 10 % to create more gentle conditions.

For runs Lingonberry-3 and -4 conditions were chosen based on the results from previous runs. Lingonberry-3 was cultivated with same operation cascade than Lingonberry-2: dissolved oxygen target was set to above 10 % by increasing first stirring from 50 rpm up to 70 rpm and then by increasing the aeration from 0.1 lpm up to 0.3 lpm. Lingonberry-4 was cultivated with even lower aeration and stirring than Lingonberry-1 to reach the oxygen limitation during growth. Stirring was set to 40 rpm and aeration to 0.05 lpm.

For batches Lingonberry-5 and -6 different light wavelengths were used. The aim was to test if the wavelength could have a strong effect on pigment formation. Lingonberry-6 had also the higher target of dissolved oxygen, 20 %, to see if it has a negative impact for biomass production.

Batches Lingonberry-7 and -8 were carried out without stirring, operating bioreactors with airlift strategy. With aeration level of 1 lpm the flow pattern was effective enough to move cells also at the lower part of the vessel. Difference between batches was the pre-autoclaving pH of the medium and the aim was to monitor its effect to biomass accumulation.

All cultivation conditions are collected for comparison to the table 4 below.

Table 4. *Cultivation conditions of stirred tank bioreactor experiments Lingonberry-1 to 8.*

	Lingonberry-1	Lingonberry-2	Lingonberry-3	Lingonberry-4	Lingonberry-5	Lingonberry-6	Lingonberry-7	Lingonberry-8
Source of inoculum	Wave+flasks	Wave+flasks	Lingonberry-2	Lingonberry-2	Lingonberry-4	Lingonberry-3	Lingonberry-6	Lingonberry-6
Target inoculum density (g/L)	60	60	60	60	60	60	60	60
DO target (%)	to reach 0	> 10	> 10	to reach 0	>10	>20	-	-
Aeration (slpm)	0.1	cascade from 0.1 to 0.3	cascade from 0.1 to 0.35	0.05	cascade from 0.1 to 0.35	cascade from 0.1 to 0.45	1	1
Stirring (rpm)	50	cascade from 50 to 70	cascade from 50 to 70	40	cascade from 50 to 70	cascade from 50 to 70	no stirring	no stirring
Amount of LED panels	1	1	2	1	2	2	2	2
Irradiance (µmol m^{-2} s^{-1})	70-90	70-90	0 h: 70-90 22 h: 200-250 43 h: 550-650 168 h: 700-800	0 h: 70-90 22 h: 200-250 43 h : 550-650 168 h: 600-700 309 h: 800-1100 330 h : 1000-1500	700-800	700-800	700-800	700-800
Illumination regime (h)	16:8	16:8	24	24	24	24	24	24
Color of light	White	White	White	White	Green-Blue	White	White	White
Pre-autoclaving pH of medium	4.8	4.8	4.8	4.8	4.8	4.8	7	4.8
Working volume (L)	2	2	2	2	2	2	2	2

3.4.4 Sampling and measurements

Samples were taken from stirred tank bioreactors daily through sampling siphon to a sterile bottle. Approximately 40 ml of sample was taken each time to ensure representativeness of the whole culture as well as possible. 15 ml of sample was filtered with Bühner filter and collected to pre-weighted 15 ml Falcon tube. Fresh weight was measured from these filtered cells. Dry weight was measured from the same sample by using the same procedure than shake flask experiments. Conductivity and pH were measured from the filtered supernatant. After pH and conductivity measurement suspension was filtered via syringe through Ø 45 µm filter for sugar consumption analysis, described in section 3.5 below. The viability of the samples was estimated during the chosen days by following the steps described in section 3.7.

Contamination tests were carried out during each cultivation to ensure the asepsis of the culture. Cultivation media was plated to Trypticase soy agar (TSA), Potato dextrose agar (PDA) and Luria broth agar (LBA) plates. Plates were incubated at room temperature over 3 days and possible colony formation was observed.

Through online measurement levels of dissolved oxygen, stirring, aeration and pH were monitored from control unit. Gas composition (CO_2, O_2, and N_2) of exhaust gasses was analyzed continuously with a Prima Pro Process mass spectrometer (Thermo Scientific, UK) to detect the possible differences in cells carbon metabolism.

3.5 Sugar consumption analysis

Sugar consumption analysis for stirred tank cultivation samples was made with High-Performance Liquid Chromatography (HPLC) analysis. Double filtered supernatant samples were prepared for the analysis by making a 5 folded dilution to 5 mM sulphuric acid.

The concentrations of sucrose, fructose, glucose and glycerol were determined by HPLC (Waters Alliance 2695 Separation Module, USA) using a Fast Acid Analysis Column (100 mm x 7.8 mm, BioRad Laboratories, Hercules, CA) linked to an Aminex HPX-87H organic acid analysis column (300 mm x 7.8 mm, BioRad Laboratories) with 5 mM H2SO4 as eluent and a flow rate of 0.3 mL/min. The maintenance temperature of used column was 35 ^{o}C. Waters 410 differential refractometer and a Waters 2487 dual wavelength UV (210 nm) detector were used for detection of peaks.

3.6 Anthocyanin content analysis

For anthocyanin content analysis 10-15 mg of freeze-dried cells were weighed and extracted with 1.0-1.5 mL of methanol (MeOH, ultra-performance liquid chromatography (UPLC) -grade) in Eppendorf tubes (10 mg/mL). The samples were shaken over 5 minutes (20 Hz) with two steel balls in a Retsch homogenizer (Germany). After 0.5 h

standing and 5 min centrifugation (10000 rpm), 0.5-1.0 mL from the MeOH extract was separated into sample vials and evaporated into dryness under nitrogen flow. The residue was dissolved into 50-100 µL MeOH (100 mg/mL) and transferred into vial inserts. Aliquots with 2 µL volume were used for ultra-high-performance liquid chromatography–diode array detector–tandem mass spectrometry (UPLC-DAD-MS) analysis on an ethylene bridged hybrid particle technology (BEH) C18 (100 mm × 2.1 mm; 1.7 µm) column. The gradient started from 5% acetonitrile in 0.1 % formic acid and achieved 90% of acetonitrile in 9 minutes at a flow rate of 0.43 ml/min. The compounds were detected with a diode array detector within a wavelength range of 210–700 nm. The MS with electrospray ionization (ESI) positive ion mode was used to enhance the identification of the compounds. Anthocyanins were quantified at 515 nm by using an external standard curve for cyaniding-3-O-glucoside.

3.7 Viability measurement and cell morphology

The viability of the samples was estimated via Fluorescein Diacetate (FDA) staining treatment. Non-fluorescent FDA molecule is hydrolyzed to fluorescent in living cells and differentiation of living cells can be observed by microscopy. Roughly 100 µl of FDA stain stock solution (0.5 % of FDA in acetone) was mixed with 5 ml of fresh cultivation media and covered from light. FDA-media-solution and sample was mixed with a 50:50 ratio with each other, normally using a total volume of 1 ml. The samples were protected from light and incubated for 5 minutes before analysis. Samples were examined under a microscope by using U-filter and UV-light. Pictures of cell viability and morphology were taken via IMAGINE MicroPublisher 5.0 RTV system (Canada). The proportion of viable cells with light green fluorescent due FDA was estimated by eye.

3.8 Single-use bag experiments of lingonberry cell culture

Single-use bag experiments were carried out by using 1 L polyethylene Amergrip® reclosable bags made by Amerplast (Amerplast, 2018). Bag in a box -approach was used to create orbitally shaken disposable bioreactor prototype. For this purpose, 1 L decanter glass with cylinder shape was chosen for external support structure.

For gas exchange bag were modified by creating a hole covered with wound tape for exhaust gasses. Air inlet was made by attaching autoclaved air filter through the plastic layer and sealed the junction with Parafilm to prevent air contamination of culture. Both modifications were made to the upper part of the bag, to create a gas exchange to the headspace of cultivation bag.

For the second batch of single-use bags adjustments to prevent the formation of dead zones were made by using Hulme Martin heat sealer (GB). Figure 6 shows sealing modifications. The aim was the better fit to 1 L decanter glass to prevent the packing of cells

to the corners of the bag or formation of the folds to the sides of cultivation bag due too wide size of the bag compared to support vessel.

Figure 6. *Sealing pattern of adjusted 1L amergrip bags for the second batch of single-use cultivation bag experiments.*

Single-use bag bioreactors were cultivated in decanter glasses located into Infors HT TR-225 bench-top orbital shaker with 120 rpm under room temperature, adjusted to 24 degrees. During second batch, mixing rate was increased from 120 rpm to 125 rpm. More suitable fit of single-use bag creates more stable wave-motion to the cultivation broth, leading to less effective mixing. To prevent sedimentation of cells, mixing rate was increased after 3 days of cultivation.

Same LED-panels from bioreactor cultivations were used to give white light from one side of the cultivation vessel. Power of panels was adjusted by light measurements to be as similar as with successful bioreactor runs. More detailed information about used conditions and experimental set up of single-use bag cultivation are presented in the table 5 and Figure 7 below.

Table 5. *Comparison of different experimental set ups for single-use bag bioreactor experiments.*

	Set up 1	Set up 2	Set up 3	Set up 4	Set up 5	Set up 6	Set up 7	Set up 8
Diameter (cm)	10	10	10	10	10	10	10	10
Liquid height (cm)	4	5	7	5	5	5	6	7
Liquid height to diameter ratio	0.4:1	0.5:1	0.7:1	0.5:1	0.5:1	0.5:1	0.6:1	0.7:1
Liquid volume (mL)	300	400	500	400	400	400	450	500
Cultivation bag	1 L Amergrip	1 L Amergrip	1 L Amergrip	1 L Amergrip, adjusted	1 L Amergrip, adjusted	1 L Amergrip, adjusted	1 L Amergrip, adjusted	1 L Amergrip, adjusted
Aeration method	To headspace	To headspace	To headspace	Sparging	Sparging	To headspace	To headspace	To headspace
Aeration (L/min)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Mixing (rpm)	120	120	120	125	-	125	125	125
Shaking throw (mm)	25	25	25	25	-	25	25	25
Illumination regime	24	24	24	24	24	24	24	24
Color of light	White	White	White	White	White	White	White	White
Irradiance (µmol m^{-2} s^{-1})	700-800	700-800	700-800	700-800	80	700-800	700-800	700-800
Target inoculum density (g/L)	60	60	60	60	60	60	60	60

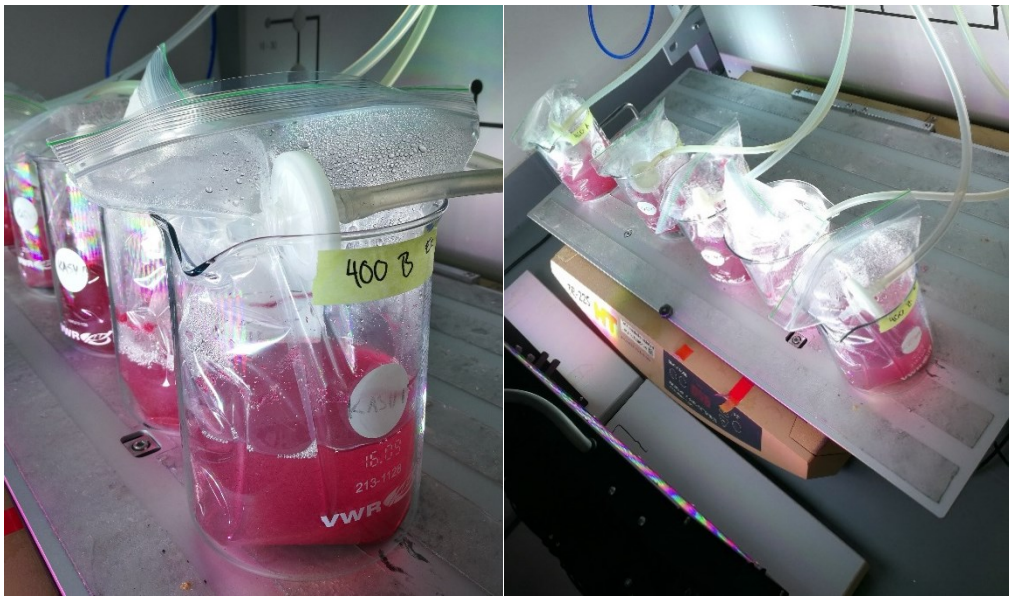

Figure 7. *Experimental set-up of single-use bag bioreactors. Reclosable plastic bags with voume of 1 L, gas inlet and outlet, and different working volumes (300-500 mL) were cultivated with orbital shaking under constant white lighting at room temperature.*

3.9 Statistics

For statistical analysis Microsoft Excel's (2016) standard deviation and standard error functions were used to describe the error of parallel samples. Statistical significance between biomass accumulations were analyzed by using T-test with two-samples assuming unequal variances.

4. RESULTS

This study evaluated how physical growth conditions affect to the biomass and color formation of lingonberry cell cultures. The aim was to study the growth of lingonberry cell cultures in a shake flask and stirred tank bioreactors to determine the critical key parameters. The hypothesis for single-use bioreactors were made based on the findings from these experiments. Finally, single-use bag experiments were carried out to verify the hypothesis.

4.1 Determination of base-line growth

Control growth curves of tobacco BY-2 and lingonberry were defined under used standard conditions to compare the growth of lingonberry under different physical growth conditions. BY-2 is used commonly in plant biotechnology.

The growth curve of tobacco BY-2 was defined by measuring fresh and dry weight, pH and conductivity from different time points from three replicas. Packed cell volume was used for fresh weight measurement. The growth curve illustrates typical growth of BY-2 culture. The decrease of conductivity correlates the increase in biomass. Value of pH is first increasing from starting point, then stabilizing around value 6 and starts to decrease towards the end of cultivation with the amount of biomass. Maximal DW, 16.8 g/L, is obtained a bit earlier than maximal fresh weight. This indicates about the more active vacuole formation at the end of cultivation. Within one week the fresh biomass of BY-2 has reached the maximal level, 645.2 g/L.

The control growth curve of lingonberry was defined by using the same methods than with tobacco BY-2 growth curve. Results are presented in Figure 8 below. In comparison to BY-2, the growth of lingonberry cells is notably slower. Maximal fresh biomass, 313.9 g/L, is achieved after 14 days of cultivation, so cultivation time is more than double compared to BY-2 cells. Also production of fresh biomass is notably lower. Difference in dry biomass formation for instance is not that remarkable. Maximal DW of lingonberry reached the level 13.9 g/L. Conductivity and pH behave alike with tobacco. Value of pH is first slightly decreasing and then growing, achieving the value near 6. After that pH is decreasing again. At the end of cultivation compounds from died and lysed cells again increase the value. Conductivity is decreasing because of sugar consumption, corresponding with biomass formation.

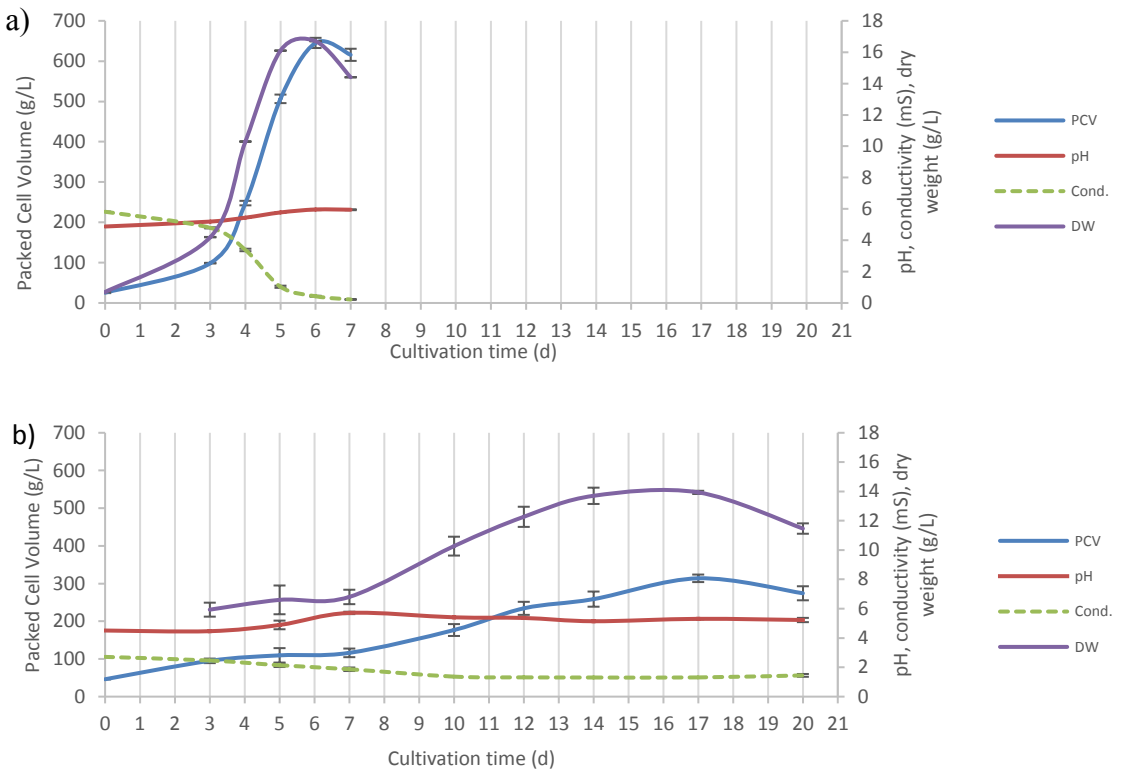

Figure 8. *Growth curve of a) tobacco BY-2 and b) lingonberry in 250 ml shake flask with a working volume of 50 ml. Biomass accumulation was observed via packed cell volume (PCV) and dry weight (DW) measurements. Conductivity and pH were analyzed. Average and standard deviation of three (n=3) replicates are shown.*

The use of packed cell volume -method for defining the amount of fresh biomass of culture is not suitable for lingonberry cultivation due to the big cell aggregate size. The error in measurement is remarkable, due difficult removal of the media. Big cell aggregates block the dense packing of cells to the bottom of the tube, forming holes and spaces for media. Cell pellet at the bottom of the tube is not compressed enough and liquid cannot be poured out easily. Further results for lingonberry are defined by using bühner filtration for fresh weight measurement. It needs to be taken into account that the filtration method provides drier cell mass and this way also in relation lower biomass formation results.

4.1.1 Inoculum density

Studies have shown that inoculum density can impact the growth rate of culture. The hypothesis was that inoculum density might affect the length of lag-phase at the beginning of cultivation. For inoculum density experiment 3 different values were used: 40, 60 and 80 g/L. All cultures with different inoculum concentrations grew well and their growth curves, pH and conductivity values are presented in a Figure 9 below.

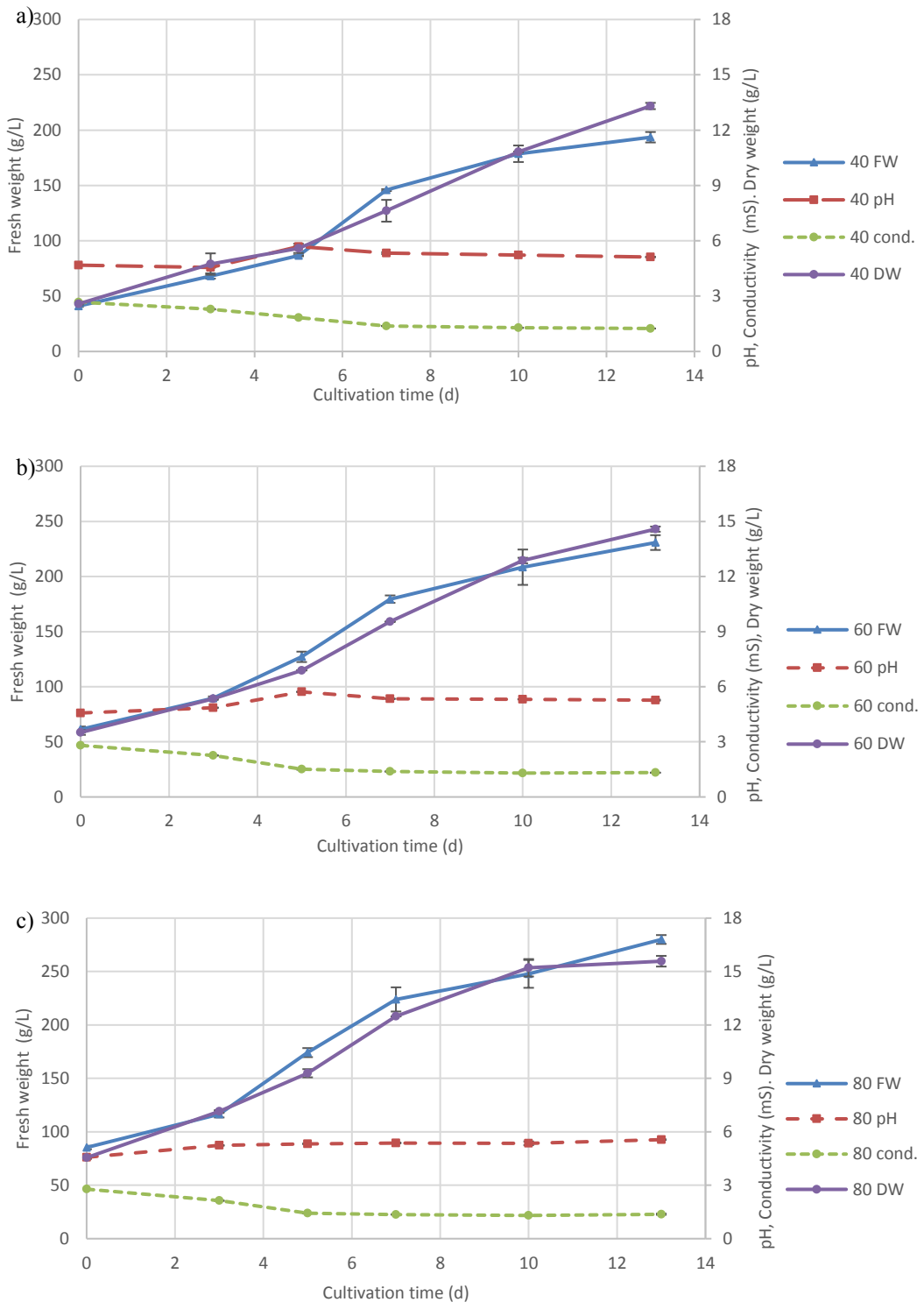

Figure 9. *The growth curves of lingonberry suspension cultures in 250 ml shake flasks with target inoculum density of a) 40 g/L, b) 60 g/L, c) 80 g/L. Biomass accumulation was observed via fresh weight (FW) and dry weight (DW) measurements. Conductivity and pH were analyzed. Average and standard deviation of two (n=2) replicates are shown.*

Each cultivation had a lag phase with slower biomass formation, followed by exponential growth phase. Comparisons of fresh and dry weight increase during cultivation are presented in Figure 10 below. With 80 g/L starting inoculum density, the length of the lag

phase is shortest, and the amount of increased biomass is, therefore, biggest, 195 g/L. With starting inoculum of 40 g/L, reached an increase of fresh weight, 153 g/L is lowest due to the longest lag phase. When only the amount of dry weight is compared, the difference between different inoculum densities is not that significant. For all the cultivations increase of dry biomass was about 11 g/L. This increase was achieved fastest with inoculum density of 80 g/L, after 10 days of cultivation. After that cells started to gain more FW probably by forming more or bigger vacuoles.

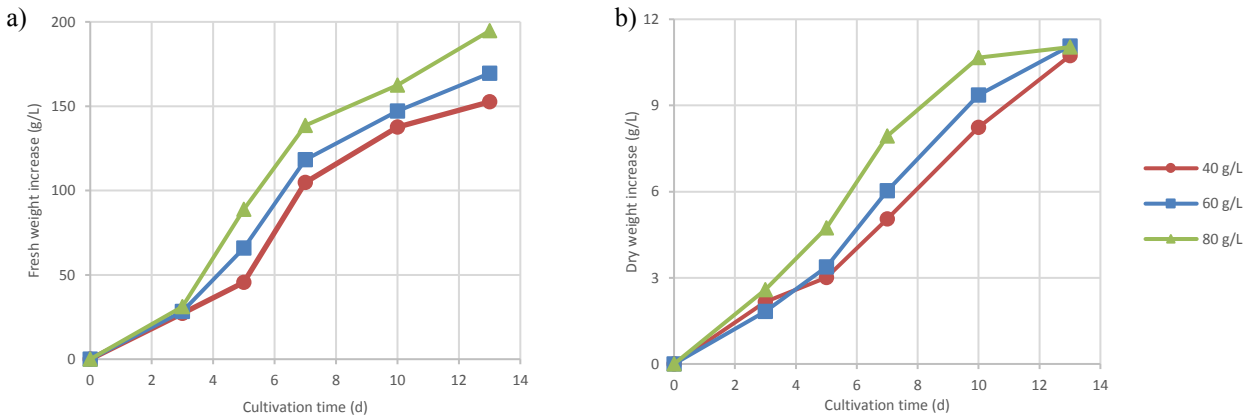

Figure 10. *a) Fresh and b) dry weight increase during growth with target starting inoculum densities of 40, 60, and 80 g/L. Increase in each time point is presented as a difference of average values of two replicates.*

It seems that higher inoculum density leads to shorter lag phase and therefore faster biomass formation. It is possible, that cultures with lower starting inoculum densities would have continued their growth still after 13 days and eventually reached the even bigger biomass increase than 80 g/L inoculum. Still, too long cultivations times are also problematic for this application. Based on these results, higher starting inoculum seems to be a more suitable option for fast biomass production.

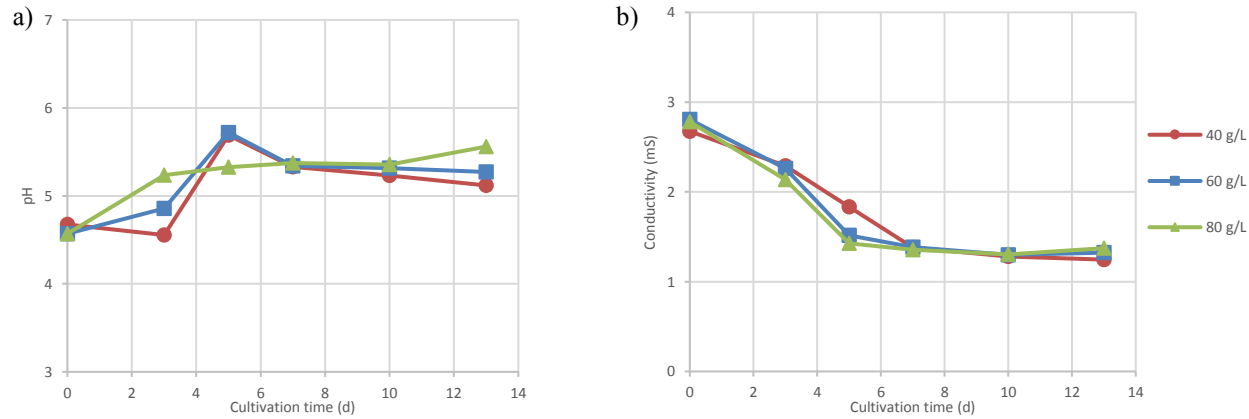

Figure 11. *a) The pH and b) conductivity difference between cultures with different starting inoculum density. Increase in each time point is presented as a difference of average values of two replicates.*

Conductivity and pH differences between each inoculum density treatments are presented in a Figure 11 above and results are in line with fresh weight formation. With the highest inoculum density typical pH value during growth phase is reached faster and conductivity decreases also rapidly. With lowest inoculum density, 40 g/L, both pH and conductivity change are lacking from other treatments, indicating also lag phase in biomass formation.

4.1.2 Gas exchange

DO level is a critical physical factor for biomass formation. Effects of gas exchange in shake flasks were tested by measuring the biomass formation after 13 days cultivation. Aim was to see, if cultures in shake flasks are limited by lack of oxygen and does it cause any visible changes to culture. Results of the gas exchange experiment are presented in a Figure 12 below.

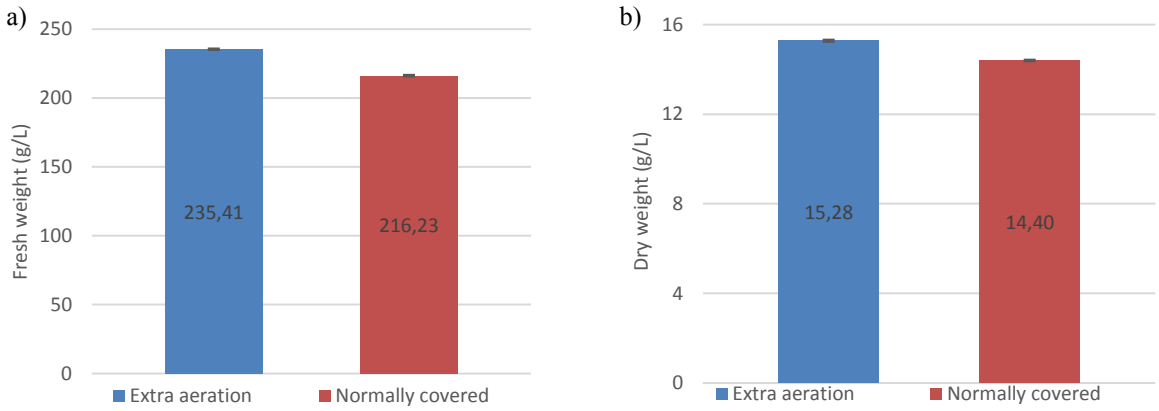

Figure 12. *a) Fresh and b) dry weight of lingonberry after 13 days of cultivation in 250 ml flask with 50 ml working volume. Average and standard deviation of replicates are shown. For extra aerated samples n=6, and for normally covered samples n=7. Difference in fresh weight (p=0.036) is statistically significant, but with dry weight (p=0.458) it is not, based on T-test.*

Both fresh and dry weight have increased when more effective aeration of cultivations is used (Fig. 12). Difference is still not statistically significant. Fresh weight of extra aerated samples is almost 9 % higher compared to normally covered samples. For dry weight, the difference is 6 %. Oxygen can be a limiting growth factor in a shake flask cultivation of lingonberry and can reduce biomass formation. Still, viability and visual appearance of both cultures were good and possible oxygen limitation did not cause any other problems. Reduction in growth was not that significant either.

4.1.3 Light conditions

The effect of light for biomass production of lingonberry was monitored through dark cultivations. The original hypothesis was that strain which was used to grow under light would suffer some damage when moved to grow under continuous darkness. The first

aim was to make a rough experiment about the cultivation of lingonberry cells in dark conditions to see if they can survive at all or not.

In a first dark cultivation experiment with 40 g/L starting inoculum density high biomass accumulation was achieved after 13 days of cultivation. Comparisons between samples cultivated under 16-8 h photoperiod and in dark are presented in a Figure 13 below.

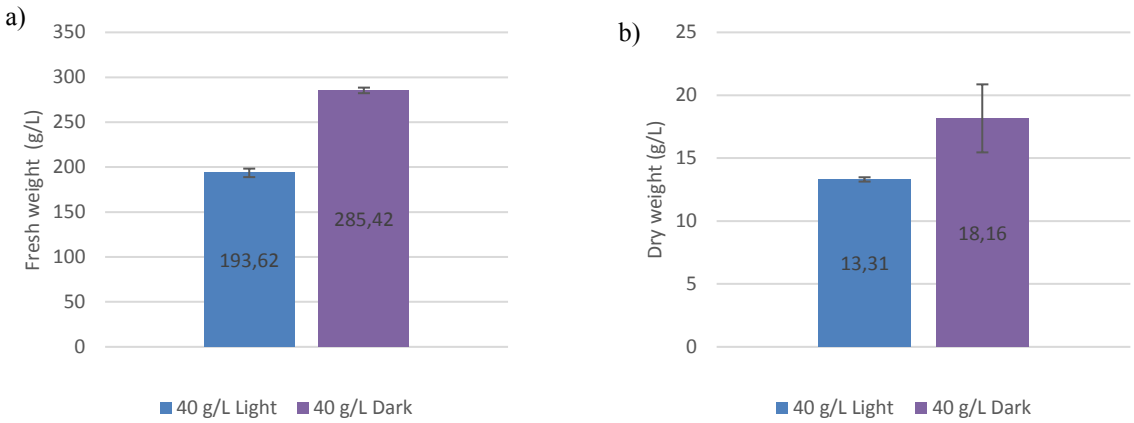

Figure 13. *a) Fresh weight (g/L) and b) dry weight (g/L) accumulation comparison of lingonberry cells after 13 days of cultivation in 250 ml shake flasks with 50 ml working volume and 40 g/L starting inoculum for cells grown under 16-8 h day-night rhythm and under continuous darkness. Average and standard deviation of two (n=2) replicates are shown. Difference in fresh weight (p=0.004) is statistically significant, but with dry weight (p=0.324) it is not, based on T-test.*

The average fresh weight results of cells cultivated in dark is almost 92 g/L and 47 % higher compared to cells cultivated under 16-8 h photoperiod. Also, average dry weigh is 36 % higher compared to light cultivated cells. Based on this experiment, cultivation of cells in dark increases the biomass accumulation. The color of cell culture was also clearly different. Cells cultivated under dark conditions lost their red pigmentation and turned to light brown/grey in color.

Due to interesting results from the first dark cultivation experiment (Dark a), more tests for dark cultivated cells were carried out. In the second dark experiment (Dark b) more detailed information about growth was studied and the growth curve of dark cultivated cells was defined. The growth curve for lingonberry cells with inoculum density of 80 g/L cultivated under continuous darkness is presented in Figure 14 below.

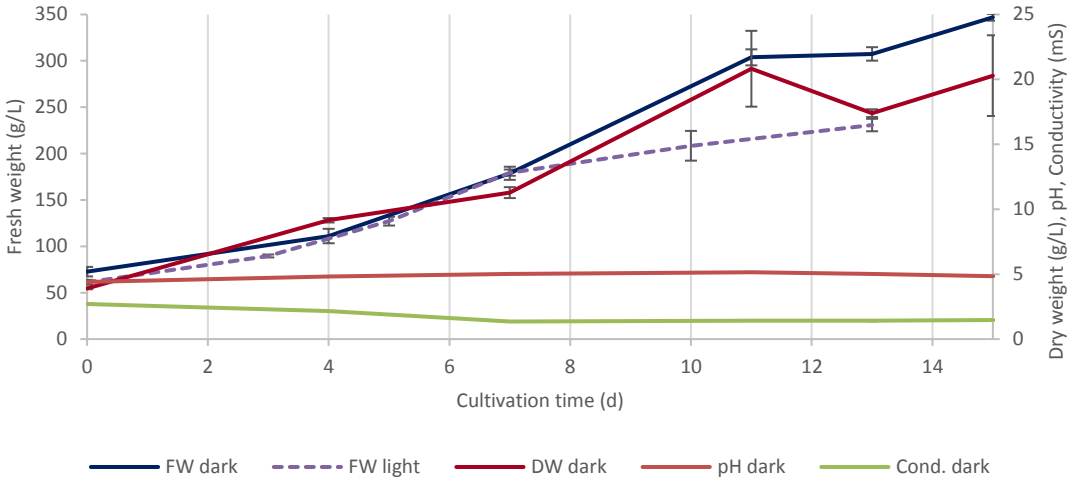

Figure 14. *Growth curve of lingonberry cultivated in dark in 250 ml shake flask with a working volume of 50 ml. Fresh weight, dry weight, pH and conductivity were monitored to analyze the growth. Fresh weight of culture grown under light is presented for comparison (results from inoculum density experiment, starting density of 60 g/L). Average and standard deviation of two (n=2) replicates are shown.*

The experiment was finished after 15 days and during this time fresh biomass accumulation reached level 347 g/L. After 13 days of cultivation amount of fresh weight was almost 30 % higher than with cells grown under 16-8 h photoperiod in previous inoculum density experiment with starting density of 60 g/L. This confirms the conclusion made from previous dark cultivation. Biomass accumulation is faster when lingonberry cells are cultivated in dark. Clear difference in growth can be seen after 7 d cultivation.

Two-stage cultivation tests were carried out due to interesting biomass accumulation results of both dark cultivation trials, but the undesired color of final cell mass. The aim of this experiment was to achieve optimal biomass accumulation in dark but still reach sufficient red pigmentation during short cultivation period under the light. More optimal conditions for each stage could be applied by using this kind of cultivation strategy.

Four different cultivation strategies were used: continuously in dark, 7 d in dark and 8 d under the light, 11 d in dark and 4 d under the light, and continuously under 16:8 h photoperiod. The biomass and color comparisons at the end of cultivations (15 days) are presented in Figures 15 and 16 below.

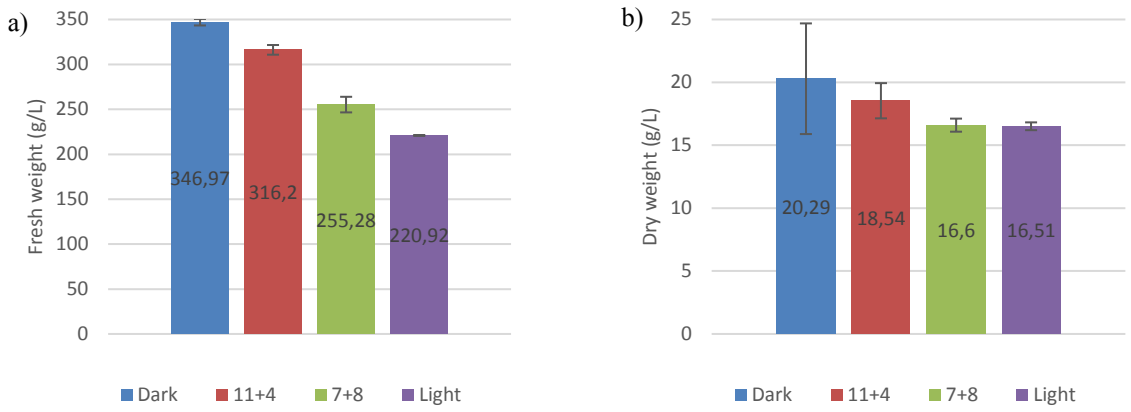

Figure 15. *a) Fresh weight (g/L) and b) dry weight (g/L) comparison of lingonberry cells after 15 days of cultivation in 250 ml shake flasks with 50 ml working volume and 40 g/L starting inoculum for cells grown continuous in dark, 2 stage cultivation with 11 d in dark and 4 d of photoperiod, 7 d in dark and 4 d of photoperiod or continuous 16-8 h photoperiod under white fluorescent light. Average and standard deviation of two (n=2) replicates are shown. Difference in fresh weight (p=0.018) is statistically significant between cells grown in dark and in light, but with dry weight (p=0.440) it is not, based on T-test.*

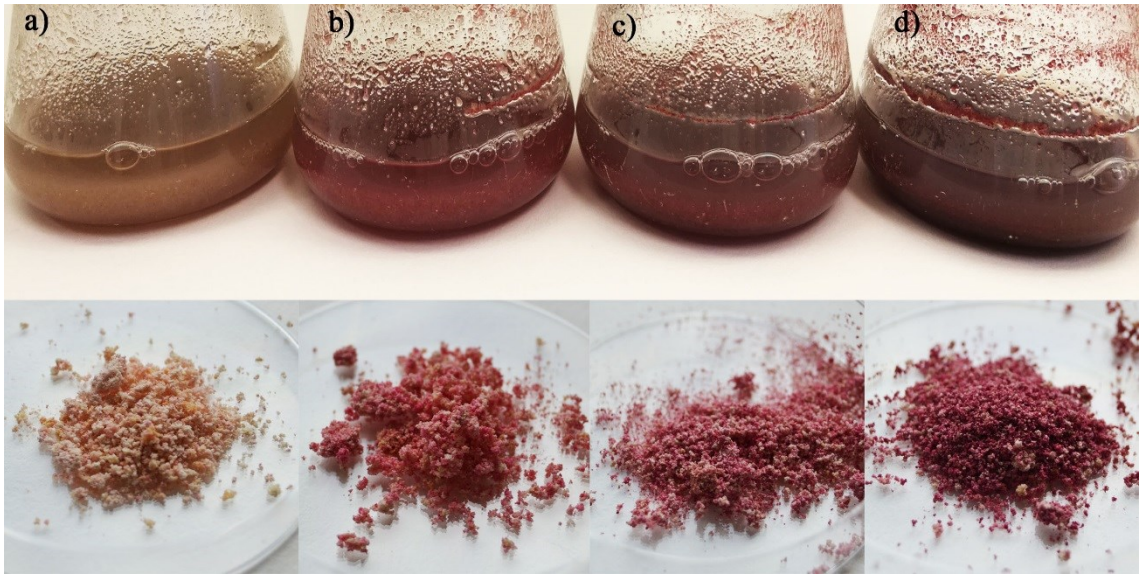

Figure 16. *Color change between lingonberry cells. Upper row after 15 days of cultivation in 250 ml shake flasks with 50 ml working volume and 40 g/L starting inoculum and lower row after freeze-drying for cells grown under a) continuous darkness, b) 2 stage cultivation with 11 d of darkness and 4 of photoperiod, c) 7 d of darkness and 4 d of photoperiod or d) continuous 16-8 h photoperiod under white fluorescent light.*

Both fresh and dry biomass were higher with cells cultivated under continuous darkness compared to cells which are cultivated even partially under the light. Based on these biomass results presented in Figure 15 and color change in Figure 16, it can be said that light is a significant factor both for anthocyanin and biomass production for lingonberry cells. Effect of light is reversible for color formation. In dark conditions cells lose their pigmentation. When they are exposed to light again, color formation restarts.

The fresh weigh for cells cultivated in dark is 57 % higher than with cells cultivated under continuous photoperiod. Difference is also statistically significant. For dry weight, the impact is not that noticeable only 23 %. This result of DW can be misleading due to a high error rate in standard deviation of dark cultivated cells.

Only cells cultivated under photoperiod through the whole time reached the dark red color. Still, both of the cultures cultivated only partially under photoperiod gained some red pigmentation at the end of cultivation, even though redness was paler.

4.2 Stirred tank bioreactor cultivations of lingonberry cell culture

Lingonberry cell cultivations were carried out in stirred tank bioreactors, to collect more detailed online data about growth parameters. Stirred tank bioreactor was chosen from other available bioreactor options because it is widely used in other studies, also with berry cell cultivations. The aim of these experiments was to iterate how different physical factors affect the growth or visual appearance of lingonberry cells. Optimal culture conditions can be more easily optimized in future based on gained results. One target was to find the critical level of DO. Also, understanding about color formation was searched, as well as cell line's tendency for foam formation. Only batch cultivation model was studied due to its suitability for Home bioreactor application.

Data from six batches of lingonberry cell cultivations in stirred tank bioreactors is presented in Figures 17 to 22 below. During first samplings from both batches Lingonberry-1 and -2, it was noticed that taking a representative sample from bioreactor can be challenging and there is some variation among measured samples. This can explain some different values of fresh and dry weight results in every stirred tank cultivation measurement points.

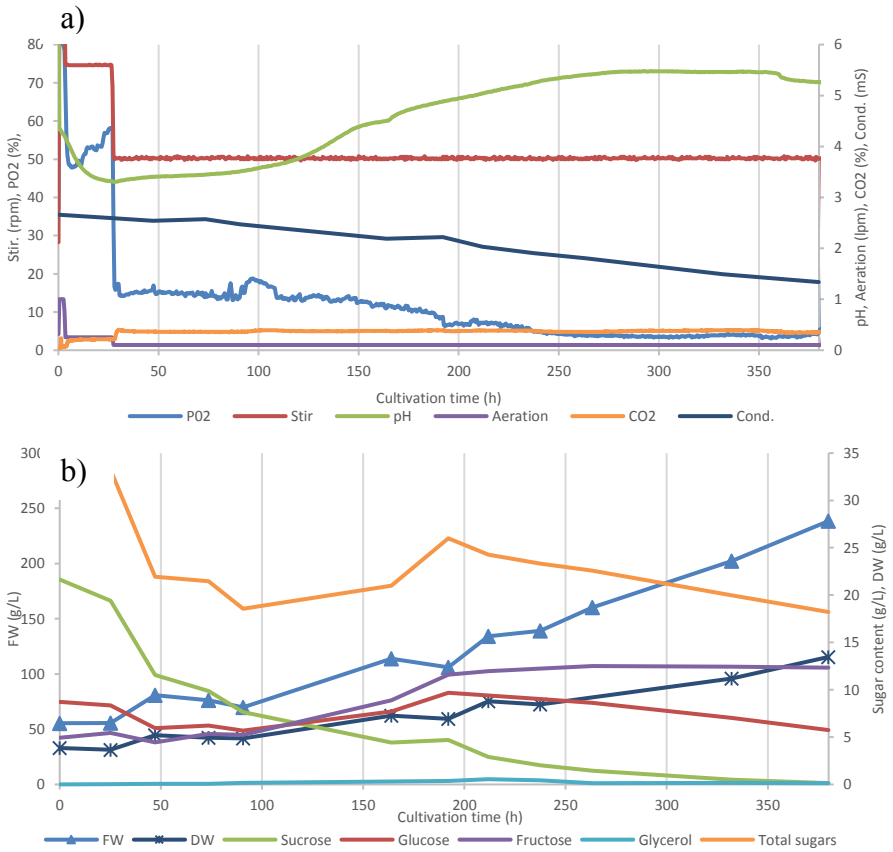

Figure 17. *Lingonberry-1 a) cultivation data and b) sugar consumption analysis from 2 L wv stirred tank bioreactor with 16:8 h photoperiod. Dissolved oxygen level (PO2), stirring, pH, aeration level, and CO_2-level from exhaust gasses were monitored online. Fresh weight, dry weight, conductivity, and sugar content were analyzed from daily samples.*

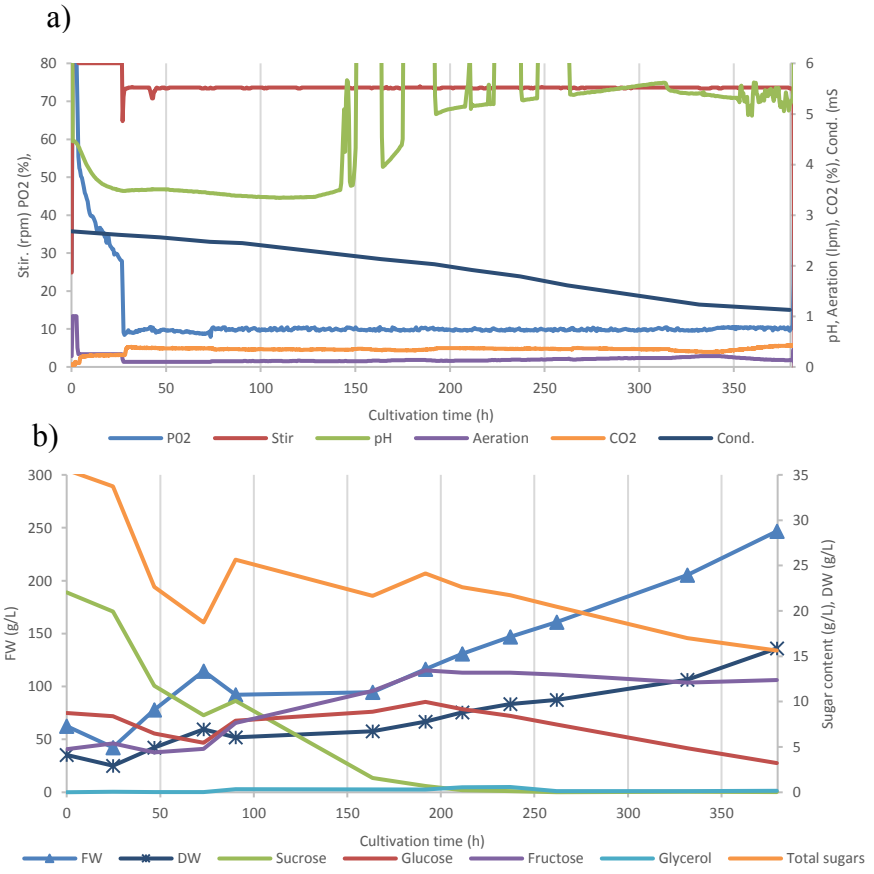

Figure 18. *Lingonberry-2 a) cultivation data and b) sugar consumption analysis from 2 L wv stirred tank bioreactor with 16:8 h photoperiod. Dissolved oxygen level (PO2), stirring, pH, aeration level, and CO_2-level from exhaust gasses were monitored online. Fresh weight, dry weight, conductivity, and sugar content were analyzed from daily samples.*

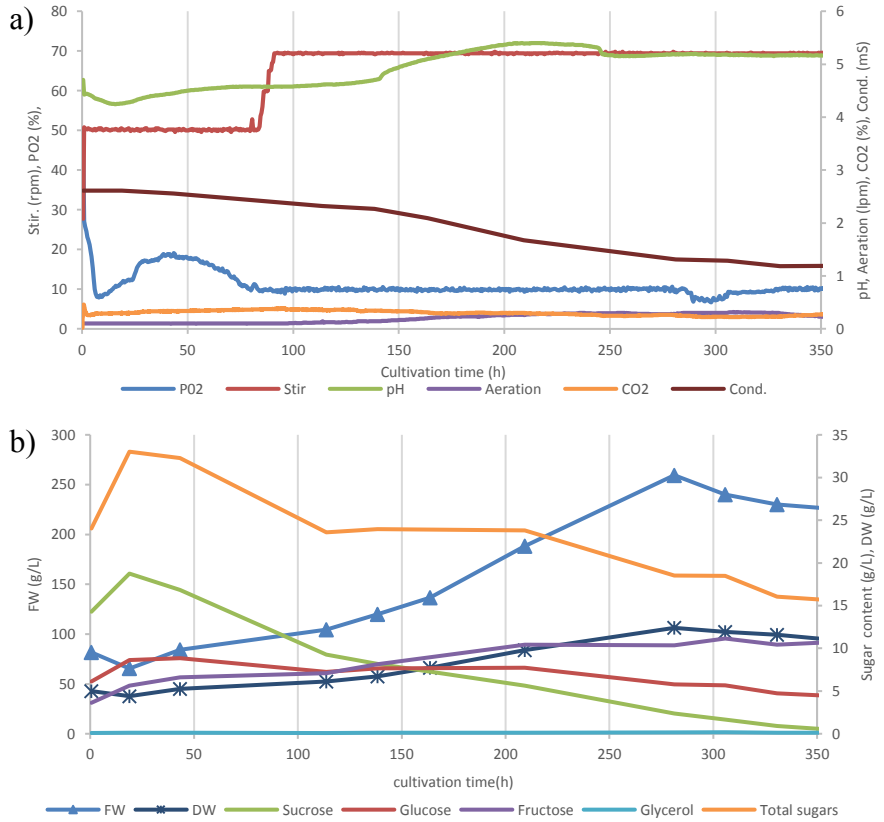

Figure 19. *Lingonberry-3 a) cultivation data and b) sugar consumption analysis from 2 L wv stirred tank bioreactor with adapted light conditions. Dissolved oxygen level (PO2), stirring, pH, aeration level, and CO_2-level from exhaust gasses were monitored online. Fresh weight, dry weight, conductivity, and sugar content were analyzed from daily samples.*

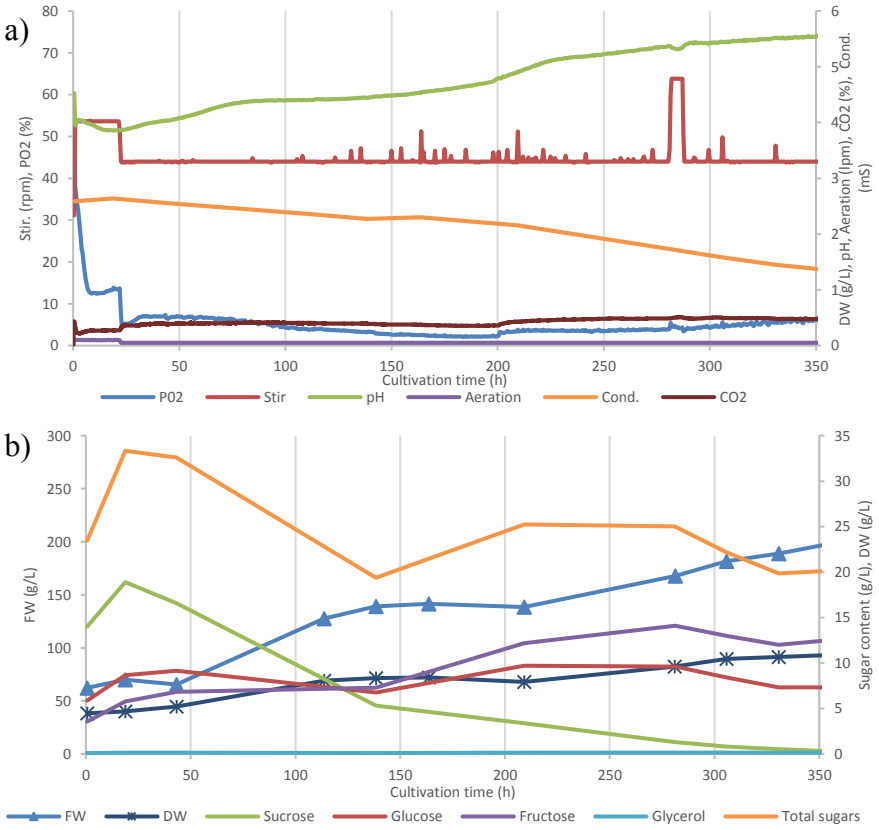

Figure 20. *Lingonberry-4 a) cultivation data and b) sugar consumption analysis from 2 L wv stirred tank bioreactor with adapted light conditions. Dissolved oxygen level (PO2), stirring, pH, aeration level, and CO_2-level from exhaust gasses were monitored online. Fresh weight, dry weight, conductivity, and sugar content were analyzed from daily samples.*

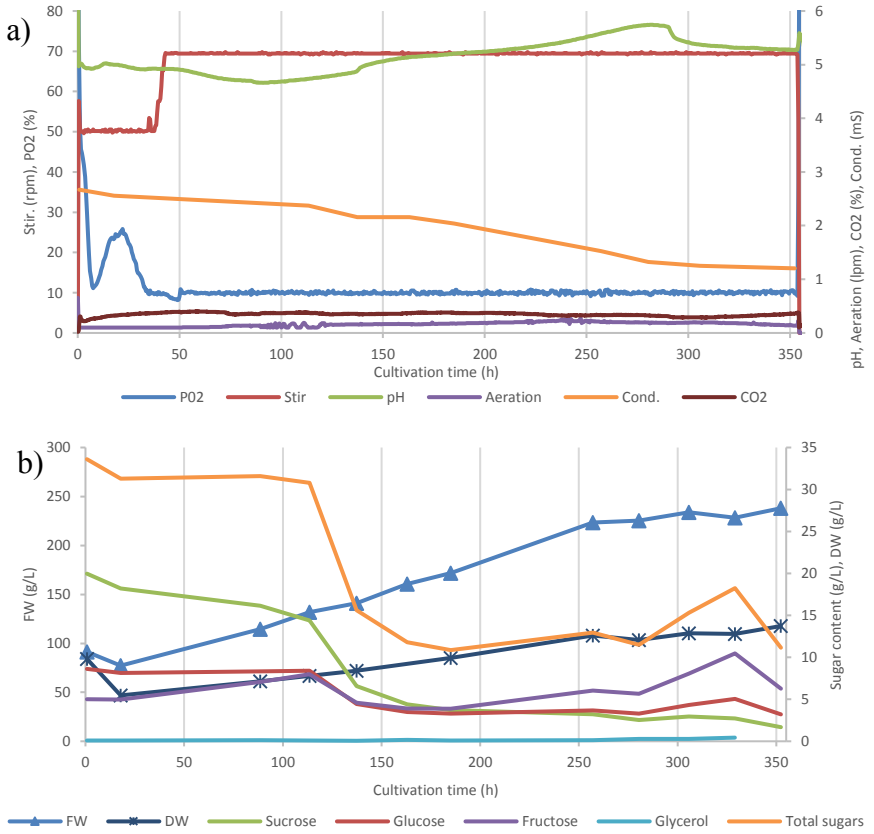

Figure 21. *Lingonberry-5 a) cultivation data and b) sugar consumption analysis from 2 L wv stirred tank bioreactor with continuous light conditions. Dissolved oxygen level (PO2), stirring, pH, aeration level, and CO_2-level from exhaust gasses were monitored online. Fresh weight, dry weight, conductivity, and sugar content were analyzed from daily samples.*

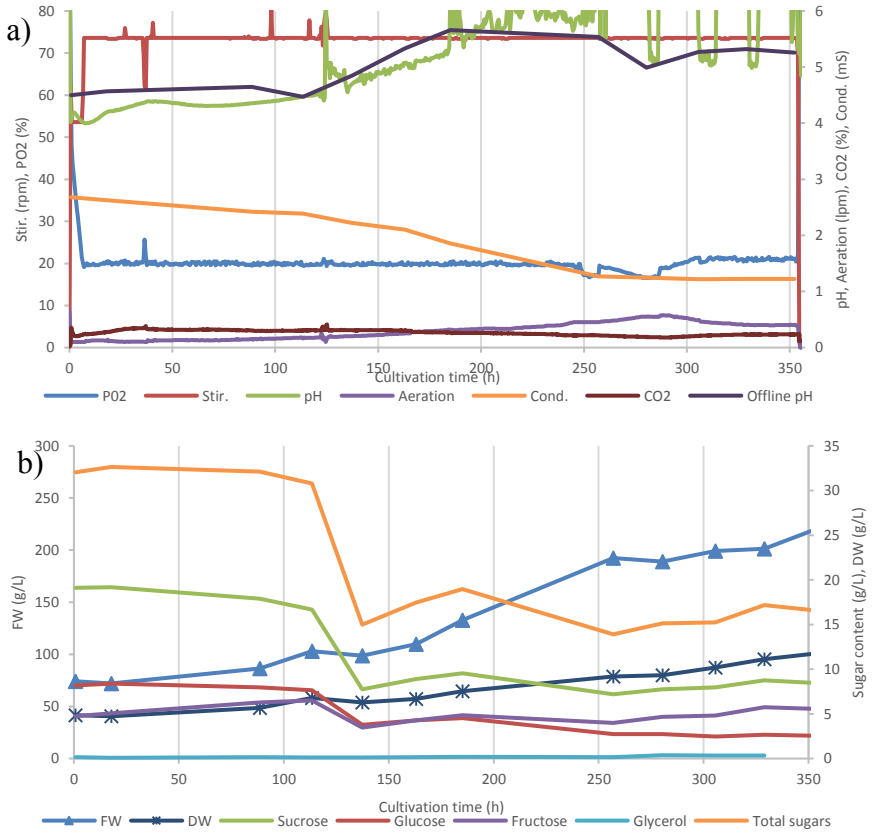

Figure 22. *Lingonberry-6 a) cultivation data and b) sugar consumption analysis from 2 L wv stirred tank bioreactor with continuous light conditions. Dissolved oxygen level (PO2), stirring, pH, aeration level, and CO2-level from exhaust gasses were monitored online. Fresh weight, dry weight, pH, conductivity, and sugar content were analyzed from daily samples.*

4.2.1 Inoculum strategy

The inoculum seems to be a critical factor affecting to the length of lingonberry cell suspension cultivation time. In batches Lingonberry-1 and -2 (Fig. 17 and 18) the inoculum cultivated in wave-bioreactor and shake flasks had difficulties to adapt to new cultivation environment with mechanical stirring. Cultures had long lag-phases, and during the first week, there was hardly any growth. The viability of cultures dropped at the beginning, when big cell aggregates broke down due to harder mixing conditions compared to seed culture. Disruption of cells caused also severe drop to pH level. After 27 cultivation hours, pH level of Lingonberry-1 culture reached the minimum value 3.06. As low values are not typical for lingonberry suspension cultures.

When inoculum for Lingonberry-3 and -4 cultivations was made from previous stirred tank bioreactor batch Lingonberry-2, lag-phases at the beginning were shorter (Fig. 19 and 20). The magnetic stirrer at the inoculation protocol caused harm and crushed some cells from inoculum. This was seen at the viability tests of culture by ruptured cells (data not shown).

Growth curves of Lingonberry-5 and -6 do not have long lag-phase (Fig. 21 and 22). Cells from previous stirred tank batch and inoculation without magnetic stirrer give fast adaptable inoculum for a new batch. Based on these results, it can be said that consistent culture conditions between the seed and target cultivation would be beneficial to prevent long lag-phase at the beginning of new cultivation.

4.2.2 Critical oxygen levels

Critical levels of oxygen consumption were searched for to study optimal growth conditions. The aim of the Lingonberry-1 batch was to reach the oxygen limitation (Fig. 17) by using low stirring and aeration levels. During this cultivation lowest level of dissolved oxygen reached the value of 3.02 % and the average level of DO after 250 h until the end of cultivation was 3.78 %. Still, there were no noticeable limitation either in fresh or dry biomass formation, which indicates that this low DO level was still sufficient for biomass accumulation at least under used light conditions. For Lingonberry-2 batch, the DO target (10 %) was maintained quite steadily (Fig. 18). At the end of cultivation, after approximately 340 h, aeration levels start to decrease even though same DO target was maintained until the end of cultivation, indicating the decreased oxygen use of culture. Otherwise batches Lingonberry-1 and -2 grow quite identically even though there was a difference in the level of dissolved oxygen. Also, batch Lingonberry-3 (Fig. 19) maintained the DO-level quite steadily at the target value (10 %). Only the drop in DO-level after 280 cultivation hours was caused by the too low upper limit of aeration cascade. DO-drop was not remarkable and probably did not have an impact on the biomass accumulation of cells.

The aim of batch Lingonberry-4 was again to reach the oxygen limitation to detect the critical level of DO for lingonberry cells. To accomplish this, even lower aeration and mixing values than in batch Lingonberry-1 were used. Based on fresh and dry weight graphs presented in Figure 20. above, it is clear that limitation in growth was achieved and it is most likely due to a low dissolved oxygen level of culture. Limitation started approximately after 140 cultivation hours when the level of DO dropped under level 3.3 %. During the next 60 hours, the average value of DO was 2.44 % and lowest reached DO-level 2.09 %. So even though there was a clear limitation in growth, DO-value never reached absolute zero level. Aeration (0.05 lpm) and mixing (40 rpm) levels were maintained constant during whole cultivation, but still, after 200 h, a clear increase in DO level was observed. Also, CO_2 level and biomass amount started to increase after 200 h, indicating perhaps some kind of metabolic change of cells. The DO level increased quite constantly until the end of cultivation, but the CO_2 level was quite stable between value 0.45 and 0.5 % of measured exhaust gasses after 250 cultivation hours.

One explanation for these results could be that cells sense too low oxygen levels and can switch to the photosynthetic pathway. Still, for making that kind of conclusions, more data should be available. But it seems clear, that when dissolved oxygen levels is lower than 3 % risk of growth limitation is present with used light conditions. In batch Lingonberry-1 DO level dropped also near 3 %, but there was no sign of growth limitations. The sufficient dissolved oxygen level might be dependent on the amount of available light. In a batch Lingonberry-4 the light intensity was higher and continuous.

Lingonberry-6 was cultivated with higher DO-target (20 %), to see if higher aeration and mixing rates can be harmful to lingonberry cell suspension culture (Fig. 22). The biomass accumulation decreased compared to batches cultivated with lower DO target (10 %). This may reflect the negative impact of higher aeration and mixing rates.

The higher shear stress caused by mixing and aeration increased also foaming problems. Foam formation reduced the number of available cells in liquid culture media. This and cell growth around vessel wall and stirrer shaft above surface level were notable during whole cultivation time. Figure 23. below shows the amount of attached cells to reactor wall after cultivation. A lot of cells were caught to this layer above the foam, which could partially explain the lower amount of biomass in a liquid suspension culture. Captured cells inside the layer were had no sufficient access to required nutrients and oxygen, and this led to the death of these cells. Foaming is normally caused by extracellular compounds secreted by cells due to high shear stress or too high sparging. Lower aeration and stirring values should be used to avoid foaming and wall growth of lingonberry cells, especially when sparging is used as an aeration method.

Figure 23. *Lingonberry-6 batch with foaming and cell growth problem. The left picture was taken at the end of cultivation, before emptying the bioreactor. Grey areas of dead cells can be seen in the middle of the attached cell layer. The right picture was taken before washing the bioreactor to illustrate the amount of attached cells.*

4.2.3 Sugar consumption

Based on the sugar consumption analysis, data shown in figures 17-22, lingonberry suspension cultures use glucose as their main energy source. Figures 17 and 18 show that during long cultivations cells can decompose all sucrose from media. They consume primarily glucose, but not available fructose. Even though growth seems to achieve its stationary phase in some cultivations at the end of cultivation, there are still available sugars and even sucrose left in medium (Fig. 21). Lack of any sugar is not limiting the cell growth at any point of any cultivation. During the oxygen-limited batch Lingonberry-4 (Fig. 20), it seems that amount of total sugars stayed almost constant between 200 and 280 cultivation hours, even though biomass accumulation restarted after 200 h. This indicates that the cells are using another energy source. One possibility is that cells started photosynthesis.

Another interesting observation from sugar consumption data is related to batch Lingonberry-6 with higher DO target. Its sugar consumption was reduced compared to batch Lingonberry-5 (Fig. 21 and 22). Also, decomposition of sucrose was slower, which indicates lower amount of cells.

4.2.4 Pigment formation, anthocyanin content and visual appearance

Anthocyanin content and pigment changes during stirred tank cultivation are presented in a table 6 below.

Table 6. *Anthocyanin content and color of freeze dried samples from the beginning and the end of each lingonberry batch cultivated in 2 L stirred tank bioreactor.*

Batch	Anthocyanin content (µg/mg DW)	
	At the beginning of cultivation	At the end of cultivation
Lingonberry-1	2.56 	0.32 
Lingonberry-2	2.56 	0.70 
Lingonberry-3	0.70 	8.17 
Lingonberry-4	0.70 	1.37 
Lingonberry-5	1.37 	20.27 
Lingonberry-6	8.17 	11.35 

Both batches Lingonberry-1 and -2 lost their red color and turned towards light brown. Reduction in anthocyanin content corresponds with color change. Despite the unpleasant color, the viability of both cultures was excellent at the end of cultivations. During batch Lingonberry-3 amount of light was increased (higher output rate and 2 LED-panels) and cells started to produce pigment again and cultivation turned back to pleasant red color. Anthocyanin content is in same level than with cell cultivated in shake flask under control conditions (data not shown). The main change from light brown color to dark red occurred between 210 and 280 cultivation hours. Increases in the amount of irradiance did not seem

to have the remarkable influence on the growth rate during cultivation. Also for batch Lingonberry-4 higher light intensity was used, but with a smaller area of the light source (only 1 LED-panel) compared to batch Lingonberry-3 and red pigmentation of culture could not be achieved during 355 h cultivation.

With batch Lingonberry-5 the formation of red pigment was studied under otherwise same kind of conditions like in batch Lingonberry-3 when cultivated cells turned to red, but instead of using white light only blue-green light was used to see if wavelength could affect somehow to the growth or pigmentation. Cells turned little by little towards red and after 160 h cultivation was equally pleasant dark red. Gained anthocyanin content reached the highest level 20.27 µg/mg DW when compared to other samples. Lingonberry-6 maintained and achieved even deeper pigmentation during cultivation than at the starting moment. Also anthocyanin content increased during cultivation. The difference between final anthocyanin concentration of Lingonberry-5 and -6 is remarkable, even though the color of both looked as dark by eye. It might be that green-blue light used for Lingonberry-5 cultivation is more suitable for anthocyanin production. Other explanation could be the more suitable mixing and aeration conditions of this cultivation, but different wavelength of light seems more logical reason.

Based on all cultivations, it seems clear that sufficient irradiance per volume of culture is needed to maintain or generate the desired pigmentation. Wavelength of used light might have great impact on anthocyanin production rate.

Batches which reached nice red pigmentation and survived from mechanical stirring were composed of from relatively small and uniformly red cell aggregates. The difference was clear in between the composition of cells cultivated in the shake flask and stirred tank bioreactor. Formation of big white aggregates, which are common in shake flask cultivations was reduced and accumulated biomass was more homogenous.

4.2.5 Air-lift approach

Batches Lingonberry-7 and -8 were cultivated without stirring to test suitability of airlift approach. Aeration was increased until cells did not settle at the bottom of the reactor. The design of the used reactor was not ideal for this kind of operation, because the aeration ring is not located directly at the bottom of the vessel. To achieve sufficient mixing, aeration level needed to be lifted up to level 1 lpm, which was almost 3 to 20 times higher than used levels in previous cultivations. This caused a remarkable increase to DO level, rising above 100 % scale. Also severe foaming occurred through both cultivations. For both batches, the shear stress caused by hard bubbling led to break down of cells. This was seen from viability measurements (Appendix C) and also from dropped pH levels. Results from batches Lingonberry-7 and -8 are presented in figure 24 below.

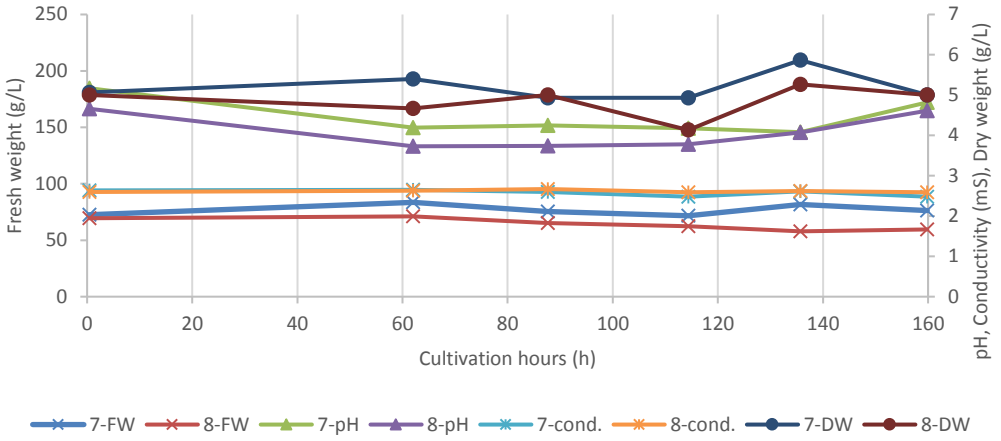

Figure 24. *Cultivation data of batches Lingonberry-7 and Lingonberry-8 from 2 L wv stirred tank bioreactor with continuous light conditions operated with air-lift approach. Fresh weight, dry weight, conductivity, and pH were analyzed from daily samples.*

As seen from figure 24, high aeration rates (1 lpm) were harmful to lingonberry suspension cell cultures. There was no growth in either batch and after 160 hours cultivations were stopped. Impact of high sparging seems to be really negative for cells. Reason can be either in shear rate created by the bubble bursting or stripping of important volatile compounds. Aeration gas contains CO_2, so stripping of ethylene could be more possible reason than lack of sufficient amount of CO_2.

4.3 Disposable bioreactor cultivations of lingonberry cell cultures

During this study lingonberry cells were successfully cultivated in the wave bioreactor and self-made single-use bioreactor.

4.3.1 Wave bioreactor

The initial aim of wave cultivations was to easy up the handling of the cell material and create a sufficient amount of biomass for stirred tank experiments. On the other hand, this trial offered a good chance to see how cells behaved in a different kind of cultivation environment and how it influenced their visual content. One of the most popular types of disposable bioreactors was tested for lingonberry cell suspension culture and it was proven that cells can be cultivated in this kind of application.

During wave cultivations lingonberry cells almost tripled their biomass during one week cultivation. Appearance of cell mass turned to paler color and bigger cell clusters formed. Color change was probably caused by non-transparent material of cultivation bag, preventing sufficient lighting. Gentle mixing enabled formation of bigger aggregates. Even

tough mixing was sensitive, some foaming and cell attachment to upper plastic layer of bag occurred.

4.3.2 Single use cultivation bag

The data from the shake flask and stirred tank bioreactor experiments were analyzed and hypotheses for single-use bag experiments were made based on the results. Aim was to apply the gained knowledge from previous experiments and create as simple and suitable single-use cultivation bag as possible.

Based on the results from mixing and aeration, single-use bag experiments were decided to carry out by using orbitally shaking application. This way bubble-free aeration with still effective mixing can be provided. Relatively big size of lingonberry cell aggregates complicates mixing by bubbling only, even though that would be the most cost-effective way to do it. Cells would easily sediment to the bottom of the bag due to insufficient mixing pattern or pack above foam layer below the surface if high sparging is used.

Due to orbitally shaking single-use bioreactor model was chosen, the shape of the cultivation bag should be either cylindrical or cubical. First, stand-alone designs were considered, but the bag in a box -application seemed then a more suitable approach. Experiments were carried out in a cylinder-shaped decanter glass by using first rectangular and then adjusted shape cultivation bag.

Material choice for single-use cultivation bag should be based on previous experiments fully transparent. This enables effective lighting. For the bag in box -application thin plastic would be most beneficial. It would smoothly take the shape of external structure. Still sufficient strength of material must be maintained to prevent breaking of bag during filling and handling. Reclosable 1 L Amergrip bags provided a good platform for single-use bag experiments in this study. The inner part of the unused Amergrip bag is readily sterile and material is thin enough.

Filling level and thus diameter to liquid height ratio, mixing rate, and wave motion inside the bag need optimization, to create suitable growth conditions for lingonberry cells in orbitally shaken single-use bioreactor. These factors affect to the level of DO and therefore biomass accumulation.

Different experimental setups were created to verify the hypotheses. The aim was to have at least one successful cultivation with lingonberry cells by using single-use plastic bag bioreactor. A working prototype of cultivation bag for the home usable bioreactor was eventually designed based on the results.

First single-use bag trial was carried out by using rectangular bags. Based on the filling level experiment for this bag type (Fig. 25), it seems that 400 mL cultivation with a liquid height to diameter ratio 0.5:1 reached best biomass productivity. Differences between 400

and 500 mL cultivation were not remarkable, but 300 mL cultivation produced notably lower amount of biomass.

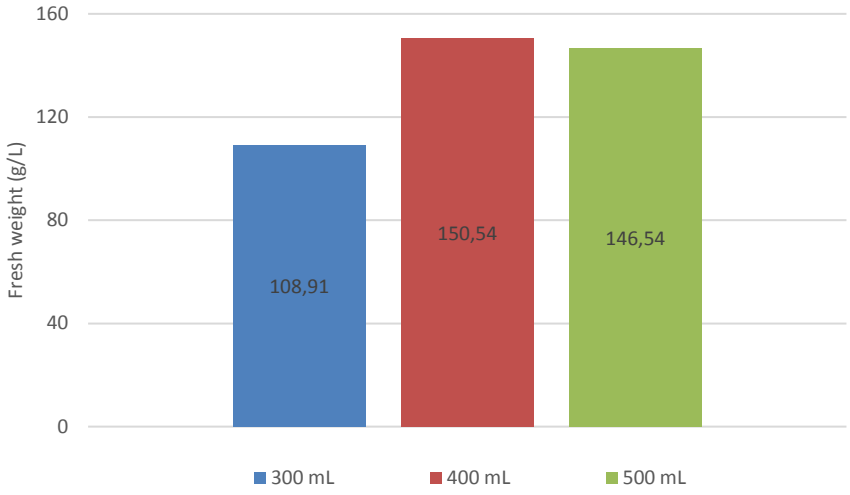

Figure 25. *Fresh weight results after 10 d cultivation in rectangular single-use cultivation bag with 120 rpm orbital shaking and 0.1 lpm aeration under continuous light. Different filling levels (300, 400, and 500 mL) were tested to optimize the level of dissolved oxygen.*

First trial revealed some problems, which should be taken into account when designing the final prototype of single-use bag. Too low filling level (300 mL) caused foaming from the beginning of the cultivation until the 5th cultivation day (Fig. 26 a). Foaming stabilized after the certain time, but wall growth and cell's attachment to plastic lasted until the end of cultivation (Fig. 26 c). It seems, that sparging is not the only factor causing foaming problems. Also, too chaotic mixing pattern can lead to unwanted foaming if too high orbital shaking speed is used together with too low filling level.

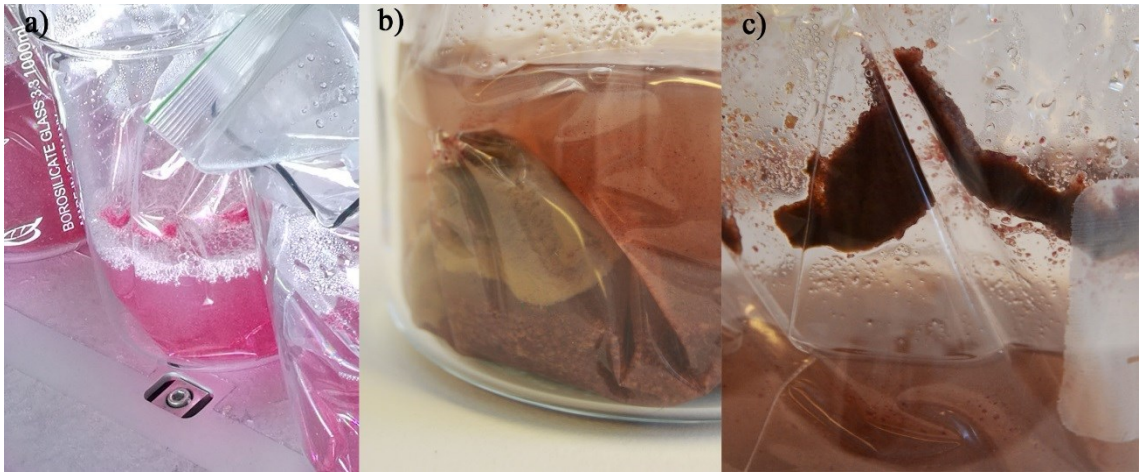

Figure 26. *Problems occurred during the first single-use bioreactor trial. a) Foaming problem of 300 mL bag at the beginning of culture. b) Captured and dead cells in the corner of the cultivation bag. c) Wall growth at the end of cultivation.*

Imperfect fitting of the bag in the supporting vessel created unwanted folds to the sides of vessel (Fig. 26 c). Folds acted as baffles and interrupted flow patterns inside the cultivation bags. Outcomes of randomly forming folds are impossible to predict. They can create chaotic mixing patterns and make it more effective or block the movement of wave totally. Folds probably caused also spilling of cultivation broth leading to the sticking of cells to bag wall (Fig. 26 c).

Inperfect fit led also to the formation of dead zones (Fig. 26 b). Corners of rectangular bag captured cells and prevented mixing of culture broth. Eventually, lack of oxygen and nutrient led to the death of the cells.

Better fitting bags for the second batch of single-use cultivation bag experiments were used to prevent the occurred problems of first trial. In an optimal situation, the shape of cultivation bag would take smoothly the shape of the outer support structure. Filling levels for second trial were chosen based on first trial. Biomass accumulation results are presented in the Figure 27 below.

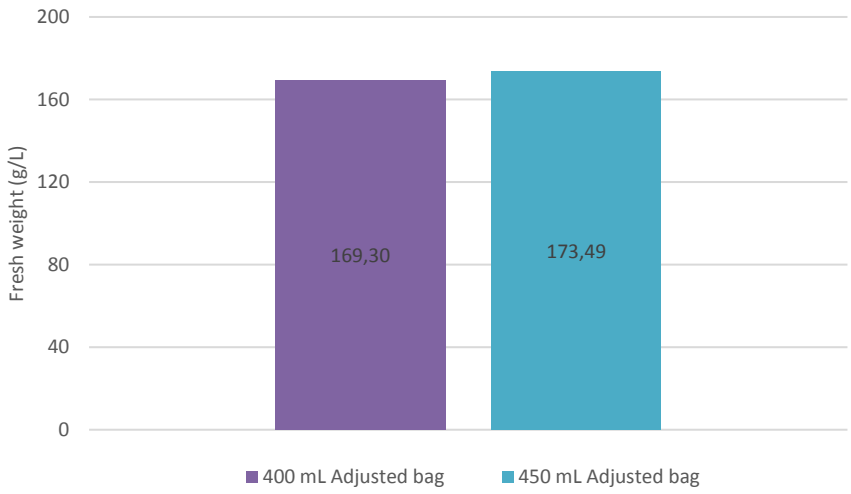

Figure 27. *Fresh weight results after 11 d cultivation in adjusted single-use cultivation bag with 125 rpm orbital shaking and 0.1 lpm aeration under continuous light. Different filling levels (400, 450 mL) were tested to optimize the level of dissolved oxygen.*

Proper fit of cultivation bag to external structure removes the baffle effect and makes mixing pattern more uniform and gentle. To maintain as effective mixing, as with rectangular bag, mixing speed needs to be increased or filling level needs to be shallower. During this experiment mixing speed was increased from 12 rpm to 125 rpm to maintain sufficient movement of cells in medium. If filling level is on the other hand too low compared to diameter and mixing speed, rotating wave motion is disturbed and wave brakes down causing chaotic flow.

Culture with 500 mL working volume was also started, but mixing (120 rpm) was not sufficient enough to prevent sedimentation of cells. Culture died and was stopped after 3 days of cultivation.

Hypothesis about negative effect of sparging was also tested by cultivating two single-use bags with sparging. One bag was mixed only by bubbling, other had both orbital mixing and sparging from the bottom. Results were in line with the hypothesis. Sparging only is not a suitable method for lingonberry cell cultivation. Culture turned brown due to inefficient mixing (Fig. 28 a). Cells sediment to the bottom and died. Also wall growth upon liquid level occurred. Culture mixed with both orbital movement and sparging as for suffered severe foaming and wall growth problems (Fig 28 b). When cells were removed manually back to cultivation medium from bag walls, lysed cells mixed with living ones, causing harm to cultivation. Also this cultivation died before harvesting. Apart of growth and foaming problems sparging causes also evaporation of liquid and during long cultivations this may result in drying of the cultivation or contamination, when air filters get moist and lose their ability to function.

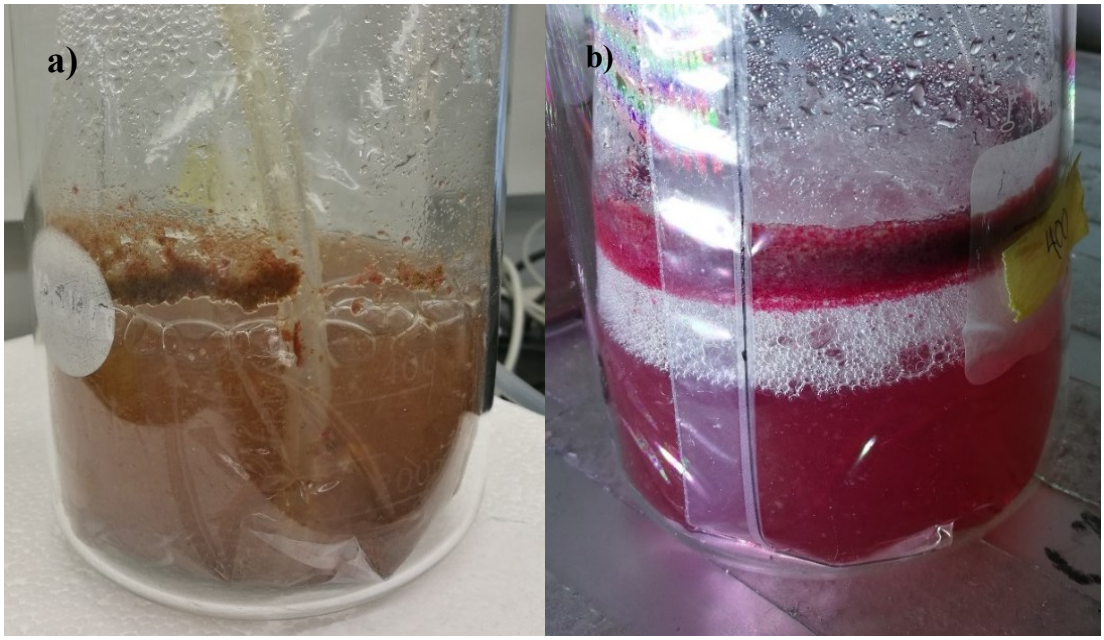

Figure 28. *Problems caused by sparging in single-use bag cultivations. a) Cultivation mixed only by bubbling turned brown after 3 days of cultivation due to inefficient mixing leading to cell sedimentation and lysis. b) Cultivation mixed with both sparging and orbital movement suffers from foaming and cell growth problems.*

5. DISCUSSION

One aim of this work was to find out what are the most relevant physical growth factors influencing biomass production of lingonberry cell suspension culture. After recognizing these factors, the second research question was how to design as a simple single-use cultivation bag for lingonberry cells as possible for Home bioreactor application?

5.1 Physical key factors for biomass production

Gained biomass production levels of lingonberry stirred tank cultivations reached satisfactory levels, even though higher biomass amounts were reached under shake flask experiments. Still, as these cultivations were the first batches made with stirred tank reactors, results were really positive. Table 7 compares produced biomass amount from successful batch Lingonberry-5 to other plant cell stirred tank cultivations.

Table 7. *Comparison of previous fresh and dry weight biomass accumulation results of different plant cell suspension cultures in stirred tank bioreactors based on literature.*

Plant species	Working volume (L)	FW (g/L)	DW (g/L)	Cultivation time (d)	Reference
Lingonberry *Vaccinium vitis-idaea*	2	230	13	14	This study*
Ohelo *Vaccinium pahalae*	10-11	117	11.8	12	(Meyer et al., 2002)
Cloudberry *Rubus chamaemorus*	300	78	-	13	(Nohynek et al., 2014)
BY-2 *Nicotiana tabacum L.*	20	600	17	7	(Reuter et al., 2014)

**Data refers to stirred tank batch Lingonberry-5.*

Based on all growing experiments it seems that some physical factors can have a significant effect on biomass accumulation and thus the success of cultivation. Suitable inoculum is a key factor for fast cultivation. If used inoculum is either too small or not adapted for upcoming culture conditions, growth is slower due to longer lag phase. My recommendation for effective growth is, that inoculumdensity should be above 60 g/L.

Oxygen usage and critical level of DO are lower than expected. Optimal DO level seems to be between 5 to 10 %, which is easy to maintain even without sparging. Maintenance of sufficient DO level can be reached by using moderate aeration and mixing levels. High sparging and also mixing can cause problems with foaming and wall growth and they

should be avoided. Cultivation by sparging only (Lingonberry-7 and -8) caused remarkable problems for cells and prevented biomass formation totally. Sufficient mixing is needed to maintain the movement of cells in suspension and to prevent the formation of cell or nutrient gradients.

During stirred tank experiments crucial foaming problem was not present in any other batch than Lingonberry-6, where DO target 20 % was used and Lingonberry-7 and -8 where only sparging was used as a mixing method. When moderate mixing and aeration levels were used, only a small amount of cells were attached to the reactor vessel. Meyer *et al.* tried to solve wall growth problem of ohelo cells by using a mechanical scraper or vortex method by periodically increasing the stirring speed, but vortex method caused harm to cells in suspension (Meyer et al., 2002). When lingonberry cells are cultivated in single-use bags, cells can be removed manually rinsing by the operator. When bigger scale cultivations are considered, the main goal should be the avoidance of foam formation rather removing it.

Mechanical stirring in bioreactor experiments provided an effective way to maintain uniform size distribution of cell aggregates. Big white clusters, which are common in shake flask cultivations, broke down and cell culture became more homogenous. This was a positive observation because white clusters are unwanted. Shake flask cultivation are constantly sieved to remove them and to maintain a culture of small red aggregates. The reason why small aggregates of lingonberry seem to produce more anthocyanin compared to big ones is unclear. Still, if the aim is to produce as much as possible deep red biomass, this feature needs to be considered. White clusters seem to grow faster and they take over easily on cell population. Unfortunately, mechanical stirring is difficult to provide for single-use bioreactors without adding unreasonable costs. Still, if this problem is considered from the point of view that consumers would cultivate lingonberry cells at the Home bioreactor, they would anyway cultivate cells from prepared inoculum and will not subculture it. In this sense problem is not that relevant for single-use bag cultivations. Still it should be taken into account in a place where the customer's seed cultures are manufactured.

Results showed that formation of deep pigmentation requires sufficient lighting. When cultivated in dark, cells do not form anthocyanin, but biomass formation increases, leading to higher fresh and dry weight result. If the aim is to produce "nice looking" biomass for food purposes, do we need the deep red color of final biomass? This should be considered because the production of partially red culture might be more cost-effective for food purposes. The light seemed to be the most important key factor for both biomass and pigment formation in lingonberry cultures, and its impact was reversible. When an insufficient amount of lighting for certain cultivation volume is used, cells lose their pigmentation, but biomass accumulation is remarkably higher. Wavelength of used light might affect significantly to the amount of anthocyanin.

Two-stage cultivation strategy could be the most suitable for lingonberry cell production due to these results. First stage in dark would create optimal conditions for fast production of biomass, but the second stage with light would be more optimal for production of the wanted secondary metabolite, which in this case is the production of anthocyanins. By adjusting the length of each stage and amount of light intensity, the balance of biomass and pigmentation could be optimized for certain applications. If high anthocyanin content is a key factor, length and intensity of light period should be increased. When higher amount of cell mass with partially red color is enough, longer darkness and less effective lighting can be chosen. Two-stage cultivation strategy could offer solutions also for bigger scale production of lingonberry. By this method, first stage of cultivation could be carried out in closed steal tank bioreactors which are easier to scale up. Then cells could be transferred for example to wave bioreactors for second stage. Then effective lighting could be provided for pigmentation.

To create better understanding about effects of light, more studies should be done. For example, optimal irradiance per cultivation volume should be iterated. Also, additional experiments of optimal wavelengths and usage of UV-light as a stimulant for color formation should be carried out. Use of green-blue light for example gave promising results and increased anthocyanin production. Other studies verify the positive effects of different light qualities to anthocyanin production (Holopainen et al., 2018), and especially responses to blue light should be investigated more in future. The optimal period or pulse for light exposure is also unclear. At least in first bioreactor trials (Lingonberry-1 and -2), where 16:8 h photoperiod was used, any clear differences between light and dark period were not noticed from e.g. exhaust gas carbon dioxide measurements. Effect of different lighting periods from continuous lightning to hour and second scale pulses should be examined. Kurata *et al.* examined this for strawberry suspension cultures, and with this species length of photoperiod impacted to anthocyanin content (Kurata et al., 2000).

This study revealed that lack of oxygen or any sugar from growth medium did not limit the growth. Limiting factor is probably some other component of the medium. If medium optimization is made at some point, amount of sugars can be reduced or search of limiting factor can be defined to other medium components. When lingonberry suspension cultures reach stationary phase before cell lysis, pH of culture starts typically decrease. Meyer *et al.* discovered that with ohelo cell culture this was caused because of exhaustion of the nitrogen source. Ohelo cell's pH curve followed the uptake rate of NH_4 and exhaustion of NO_3 (Meyer et al., 2002). Nitrogen might also be the limiting factor in lingonberry cultures. Studies have shown, that nitrogen optimization from culture media with BY-2 increased the production significantly (Häkkinen et al., 2018; Holland et al., 2010) and it should be done for lingonberry media also.

5.2 Single-use cultivation bag and Home bioreactor prototype

The development of single-use cultivation bag for the Home bioreactor was started already before carrying out this study. The functionality of the bag was still lacking from the desired level. Original idea focused on simple rectangular bag including a seamed channel for bubbling. All cultivation experiments in different bag prototypes with lingonberry cells failed but the reason behind this was unclear. This pointed out that there were multiple unknown things related to the cultivation of lingonberry cells, which needed to be solved before it would be possible to design the optimal cultivation bag.

After my investigation of the effects of physical culture conditions on the growth of lingonberry cells, decisions about single-use bags and the Home bioreactor can be made by using valid data. Reasons behind previous failures are now clear and requirements of cells are better understood. Single-use trials of this study proved, that we can successfully cultivate lingonberry suspension culture in the simple single-use bags. The biomass accumulation in simple single-use bioreactor was in sufficient level compared to biomass production after 11 d cultivation either in shake flask or stirred tank bioreactor experiment. Table 8 shows the comparison of fresh biomass production. Gained result is really satisfied, because it was reached with so simple technology.

Table 8. *Comparison of fresh biomass accumulation in different cultivation systems used in this study after 11 d of cultivation.*

Experiment	Inoculum density (g/L)	Working volume (L)	FW (g/L)	Cultivation time (d)
Adjusted single-use bag	60	0.45	173.5	11
Shake flask, inoculum density experiment	60	0.05	208.4	11
Stirred tank bioreactor, Lingonberry-5	60	2	210	11

Orbitally shaking application with bubble-free aeration is suitable for lingonberry cells. Their tendency to form heavy aggregates prevents usage of more delicate mixing. All experiments also confirmed the unsuitability of cost-effective mixing by sparging only. Selection of this orbital mixing method defined also the shape options of the bag.

Bag in a box approach was chosen above others due to its suitability for cylinder cultivation vessel. Well-fitting single-use bag is essential for these applications to prevent the formation of harmful dead-zones and folds. Badly folded plastic layers at the sides of the vessel can also prevent the correct mixing pattern and reduce the production yield. The sealing pattern used in the second trial of single-use bags might be too complex for cost

effective manufacturing of the bags. Assessments of possible industrial production sites and their production lines determine eventually the final sealing pattern of cultivation bag.

To prevent the possible contamination of cultivation bag when operated by the customer in a home environment, not by laboratory professional under sterile laminar flow, everything which goes into the bag there should go through the sterile filters. This means that needed media components and inoculum cells must be inside a bag, possibly inside another breakable plastic pocket or separated by certain folding of empty bag, when the consumer gets it. Sufficient amount of water needs to be added at the beginning of home cultivation through a sterile filter. Sterile filters are also required for inlet and outlet air. In optimal situation water and air inlet would use the same filter to make the bag as simple as possible. In this case, we can face problems with low air permeability of wet filter. Idea should be tested by using different air pressures to ensure the sufficient aeration. Also, the evaluation of commercially available options of different cap and filter options is needed for the final design of bag.

Different plastic qualities with various gas permeability rates or possible leachable components and their impact to cell growth were not examined in this study, but like mentioned during theoretical background section, this is an important part of at least the auditioning process of the product. A general observation which should be taken into account during selection of bag material are that it should be fully transparent and thin enough. This way proper light irradiance can be achieved, and the bag can easily take the correct shape of the support structure. Inner material of bag should be slippery, to avoid wall growth of cells.

From point of view of Home bioreactor design, sufficient availability of light can be achieved by choosing transparent material for cultivation bag as mentioned above and by using as unblocking support structures as possible. Normally cardboard boxes are used in bag in a box applications, but for this application it is unsuitable. My proposal is to integrate the support structure to the shaking platform of Home bioreactor. Fully transparent plastic material like acrylic would be the optimal choice for it.

Lighting can be built inside the bioreactor by using LED-technology. The original idea of designer behind the first prototype of Home bioreactor was to use it also as a light source for example to herbs growing at the kitchen table. Light was provided in this prototype from the bottom and consumer can observe the growth of cells through the partially transparent outside structure of Home bioreactor. Now, after these results, it seems that lights should surround the cultivation bag. The scale of cultivation vessel in the home environment will probably be so moderate, 0.5-1 L working volume, that if LEDs are positioned all around the cultivation bag (360^{o}), enough light can be created even though quite small LEDs need to be used due to limited room inside the bioreactor. If this approach is used for creating sufficient lighting, original idea cannot be carried out. One

solution could that light is provided only from one side of cultivation vessel and other side of it is transparent and free for observation. One option is also to provide light from the top and bottom of the cultivation vessel. For wiring, stable and not moving location for LEDs would be more optimal. Also possible heat creation of LEDs needs to be considered, especially if they are in contact with cultivation vessel.

For further Home bioreactor prototype development also design of other instrumentation inside the appliance are required. Shaking platform is needed to create the orbital mixing for cultivation bag. Engineering parameters such as shaking throw, liquid distribution and power input should be optimized for it. Normally shaking throw of 25 mm is used for applications from 25 mL up to 2 L (Infors, 2018). To find out the optimal mixing model for cell growth with as minimal power input, different shaking throws with different mixing speeds should be tested. Also pumping system for gas exchange and water transfer is required. Probably either piston pump using the movement of orbital shaker or simpler peristatic pump is chosen for the final prototype.

Proposal of suitable instrumentation for Home bioreactor was made based on these results and findings. Technical drawer Antti Niemi from Bestdan oy made a prototype model from my proposal and working prototype for further experiments can be manufactured. Design of the Home bioreactor shaking platform is presented in a Figure 29 below.

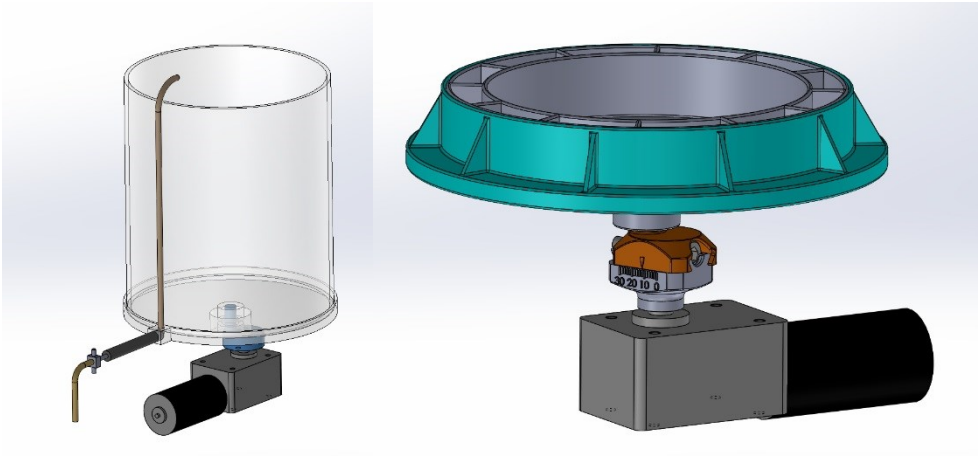

Figure 29. *Model of shaking platform prototype for Home bioreactor made by Antti Niemi from Bestdan oy. Prototype consist from orbital shaker part with stepless adjustment for shaking throw (0-30 mm) and shaking platform for two different size support cylinders (turquoise for 2 L and inner grey 1 L).*

After prototype of shaker arrives, different diameter to liquid and bag height rations with different shaking throws and shaking speeds can be optimized. After this final dimensions for cultivation bag for lingonberry cell cultivation can be selected. Detailed data about demands for suitable single-use cultivation bag can now be send to global supplier of packaging solutions for food products. With them, cost effective solution for final prototype can be designed and manufactured based on the important features of cultivation bag discussed in this work.

6. CONCLUSIONS

The ambition behind this study rose from increasing demand for novel food production technologies. The idea of sustainable, safe and self-grown food by utilizing plant cells as an edible material could be one of the futures solutions. The target of this study was to investigate more the opportunities of a simple bioreactor to grow plant cells for food in the non-laboratory environment. Impacts of different physical factors on the growth of lingonberry cells was first studied to understand their demands. Based on the results different prototypes of simple single-use cultivation bags were tested and the prototype was designed.

Experimental trials of this study showed that there are some key physical factors affecting biomass production of lingonberry cell suspension cultures. Inoculum should be suitable and adapted for upcoming culture conditions. Inoculum density should be high enough to prevent long lag phase at the beginning of cultivation. The recommended level is between 60-80 g/L. The critical level of dissolved oxygen seemed to be with used light conditions around 3 %. Based on this result and foaming problems related to higher DO levels (20 %), optimal DO level for lingonberry cell culture should be between 5 to 10 %. Moderate aeration should be used to reach this suitable DO level. Sparging causes foaming and growth limiting problems and should be therefore avoided. Mixing by mechanical impeller helps to maintain the desired small and uniform size distribution of cell culture. When mechanical mixing is not possible to use, sufficient level of mixing is needed. Sedimentation of cells to the bottom of the reactor causes cell lysis and is extremely harmful for lingonberry cell cultivation. On the other hand, due to the relatively big size of lingonberry cell aggregates and their sensitivity, too rough mixing causes growth limitations and foaming as well.

From all physical factors, the light seemed to have the greatest effect on biomass production level. When cells are cultivated under continuous darkness, highest biomass propagation can be achieved. Fresh biomass accumulation was 57 % higher with cells cultivated constantly in dark than cells grown under 16:8 h photoperiod. The light is also in a significant role for pigment formation and its effect is reversible. When cells are cultivated under darkness, red pigmentation is fading away and cells turn light brown. Under bright light red pigmentation can be regained and maintained. If only partially red biomass would be suitable for future applications, two-stage cultivation with dark and light phase is recommended. Via this approach suitable biomass-pigment ratio can be optimized by adjusting the length of each phase. Also, the amount of irradiance per cultivation volume and used wavelength influences to the deepness of pigmentation. When cultivation volumes increases, sufficient light irradiance can be challenging to achieve.

Main conclusions about important culture conditions of lingonberry are collected to a list below.

- **Use adapted inoculum for selected culture conditions**; Inoculum made from shake flask cultures caused a long lag-phase when transferred to culture conditions with higher shear stress caused by different mixing method
- **Suitable DO-target is between 5-10 %**; lower DO levels may cause growth limitation
- **Use moderate sparging**; too hard sparging causes unwanted foaming and growth problems
- **Sufficient mixing helps to maintain small aggregate size**; especially mechanical mixing was useful for maintaining the small and uniform size distribution of lingonberry culture. Sufficient mixing is also needed to prevent sedimentation.
- **Light conditions have a reversible effect on biomass and color formation**; based on experiments, a sufficient amount of irradiance is needed to reach and maintain the wanted dark red color of lingonberry culture. Higher biomass results can be achieved when cells are cultivated in dark.

When requirements of lingonberry cells were better understood based on the gained results, orbitally shaken bag in a box -type of single-use cultivation bag was designed. By orbital mixing bubble-free aeration can be achieved through head-space and foaming problems can be prevented. The effectiveness of mixing and hence level of DO can be adjusted by altering the shaking diameter, mixing speed, filling level and shape of cultivation bag. To create a systematic and predictable flow pattern, well-fitting single-use bag for external support structure should be used. In these experiments, a cylinder support vessel with adjusted cultivation bag achieved the best production rates. The material of cultivation bag should be fully transparent to enable sufficient light passing through it. Thin plastic favors better fit to the external support structure, but the strength should be high enough to prevent the breakage when the bag is operated by the customer. Single-use cultivation bag needs to include also sterile filters for water and air inlets and for exhaust gasses as well as harvesting cap with a possible filter. Inoculum and all other components needed for cultivation must be inside the bag already when it arrives to the customer, to avoid contamination risk. The bag cannot be opened before harvesting.

Final prototype was designed based on the results from single-use bag experiments and conclusions from physical culture condition trials. This model can be now used for further development work. It can also be used for cultivation of other plant cell species like cloudberry or tobacco cells.

Even though lingonberry cells were successfully cultivated in single-use bags, a lot of additional work needs still to be done. Biomass production rates were lower than with shake flask and stirred tank bioreactor cultivations and further optimization of cultivation conditions are needed. This includes for example optimization of flow pattern inside a

bag by optimizing the shaking throw and final dimensions of cultivation platform. Studies about optimal lighting conditions should be definitely carried out to understand most suitable amount, timing, quality and way of providing it for lingonberry cells. Also media optimization, probably by altering the nitrogen amount is recommended.

7. REFERENCES

Abdullah, M.A., Ariff, A.B., Marziah, M., Ali, A.M., Lajis, N.H., (2000). Strategies to overcome foaming and wall-growth during the cultivation of *Morinda elliptica* cell suspension culture in a stirred-tank bioreactor. Plant Cell, Tissue and Organ Culture, Vol. 60, pp. 205–212.

ABEC Custom Single-Run Scalable Solutions [WWW Document], (2018). URL http://www.abecsingleuse.com/ (accessed 3.6.18).

Alexandratos, N., Bruinsma, J., (2012). The 2012 Revision World agriculture towards 2030/2050: the 2012 revision.

AlgaeLab, (2018). AlgaeLab [WWW Document]. URL http://www.algaelab.org/ (accessed 7.12.18).

Amer, M., Ramsey, J.D., (2018). Multi-chamber single-use bioreactor– A proof of concept prototype. Biochemical Engineering Journal, Vol. 130, pp. 113–120.

Amerplast, (2018). Amerplast | Amerplast Amergrip bags [WWW Document]. URL http://www.amerplast.com/retail/amergrip-bag/ (accessed 7.4.18).

Andrews, G.R., Roberts, S.C., (2017). Bioprocess engineering of plant cell suspension cultures. In: Applied Bioengineering. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, pp. 283–326.

Appelhagen, I., Wulff-Vester, A.K., Wendell, M., Hvoslef-Eide, A.-K., Russell, J., Oertel, A., Martens, S., Mock, H.-P., Martin, C., Matros, A., (2018). Colour bio-factories: towards scale-up production of anthocyanins in plant cell cultures. Metabolic Engineering, Vol. 48, pp. 218–232.

Biddington, N.L., (1992). The influence of ethylene in plant tissue culture. Plant Growth Regulation, Vol. 11, pp. 173–187.

Brecht, R., (2009). Disposable bioreactors: maturation into pharmaceutical glycoprotein manufacturing. Springer, Berlin, Heidelberg, pp. 1–31.

Cellexus, (2018). Products | CellMaker [WWW Document]. URL http://www.cellexus.com/products/cellmaker (accessed 6.26.18).

Chalker-Scott, L., (1999). Environmental significance of anthocyanins in plant stress responses. Photochemistry and Photobiology, Vol. 70, pp. 1–9.

Chan, L.K., Koay, S.S., Boey, P.L., Bhatt, A., (2010). Effects of abiotic stress on biomass and anthocyanin production in cell cultures of *Melastoma malabathricum*. Biological Research, Vol. 43, pp. 127–135.

Chattopadhyay, S., Farkya, S., Srivastava, A.K., Bisaria, V.S., (2002). Bioprocess considerations for production of secondary metabolites by plant cell suspension cultures. Biotechnol. Bioprocess Eng, Vol. 7, pp. 138–149.

Davies, K.M., Deroles, S.C., (2014). Prospects for the use of plant cell cultures in food biotechnology. Current Opinion in Biotechnology, Vol. 26, pp. 133–140.

Deroles, S., (2008). Anthocyanin biosynthesis in plant cell cultures: A potential source of natural colourants. In: Anthocyanins. Springer New York, New York, NY, pp. 108–167.

Doran, P.M., (1999). Design of mixing systems for plant cell suspensions in stirred reactors. Biotechnology Progress, Vol. 15, pp. 319–335.

Doran, P.M., (2013). Bioprocess engineering principles. Elsevier/Academic Press.

Duchefa Biochemie, (2018a). McCown Woody Plant medium including vitamins | Duchefa Biochemie [WWW Document]. URL https://www.duchefa-biochemie.com/product/details/number/M0220/name/mccown-woody-plant-medium-including-vitamins (accessed 9.13.18).

Duchefa Biochemie, (2018b). Murashige & Skoog medium | Duchefa Biochemie [WWW Document]. URL https://www.duchefa-biochemie.com/product/details/number/M0221 (accessed 9.13.18).

Ducos, J.P., Pareilleux, A., (1986). Effect of aeration rate and influence of pCO2 in large-scale cultures of *Catharanthus roseus* cells. Applied Microbiology and Biotechnology, Vol. 25, pp. 101–105.

Edahiro, J., Seki, M., (2006). Phenylpropanoid metabolite supports cell aggregate formation in strawberry cell suspension culture. Journal of Bioscience and Bioengineering, Vol. 102, pp. 8–13.

Eibl, R., Eibl, D., (2006). Design and use of the wave bioreactor for plant cell culture. In: Plant Tissue Culture Engineering. Springer-Verlag, Berlin/Heidelberg, pp. 203–227.

Eibl, R., Eibl, D., (2008). Design of bioreactors suitable for plant cell and tissue cultures. Phytochemistry Reviews, Vol. 7, pp. 593–598.

Eibl, R., Kaiser, S., Lombriser, R., Eibl, D., (2010). Disposable bioreactors: the current state-of-the-art and recommended applications in biotechnology. Applied Microbiology and Biotechnology, Vol. 86, pp. 41–49.

Eibl, R., Löffelholz, C., Eibl, D., (2011). Single-use bioreactors-an overview. In: Single-Use Technology in Biopharmaceutical Manufacture. John Wiley & Sons, Inc., Hoboken, NJ, USA, pp. 33–51.

Eibl, R., Meier, P., Stutz, I., Schildberger, D., Hühn, T., Eibl, D., (2018). Plant cell culture technology in the cosmetics and food industries: current state and future trends. Applied Microbiology and Biotechnology, pp. 1–15.

Eibl, R., Werner, S., Eibl, D., (2009). Disposable bioreactors for plant liquid cultures at litre-scale. Engineering in Life Sciences, Vol. 9, pp. 156–164.

Eppendorf, (2018). BioBLU® c Single-Use Vessels - Vessels, Bioprocess - Eppendorf [WWW Document]. URL https://online-shop.eppendorf.com/OC-en/Bioprocess-44559/Vessels-66270/BioBLUc-Single-Use-Vessels-PF-157954.html (accessed 6.26.18).

Fazal, H., Abbasi, B.H., Ahmad, N., Ali, S.S., Akbar, F., Kanwal, F., (2016). Correlation of different spectral lights with biomass accumulation and production of antioxidant secondary metabolites in callus cultures of medicinally important *Prunella vulgaris* L. Journal of Photochemistry and Photobiology B: Biology, Vol. 159, pp. 1–7.

Fremont, M., (2017). Cell culture: an innovative approach for production of plant actives [WWW Document]. New Food,. URL https://www.newfoodmagazine.com/article/33682/cell-culture-plant-actives/ (accessed 8.13.18).

Furusaki, S., Takeda, T., (2017). Bioreactors for plant cell culture. In: Reference Module in Life Sciences. Elsevier.

Gao, J., Lee, J.M., (1992). Effect of oxygen supply on the suspension culture of genetically modified tobacco cells. Biotechnology Progress, Vol. 8, pp. 285–290.

GE Healthcare, (2018). ReadyToProcess WAVE 25 Rocker - GE Healthcare Life Sciences [WWW Document]. URL https://www.gelifesciences.com/en/us/shop/cell-culture-and-fermentation/rocking-bioreactors/systems/readytoprocess-wave-25-rocker-p-05542 (accessed 6.26.18).

George, E.F., Hall, M.A., Klerk, G.-J. De, (2008a). Plant tissue culture procedure - background. In: Plant Propagation by Tissue Culture. Springer Netherlands, Dordrecht, pp. 1–28.

George, E.F., Hall, M.A., Klerk, G.-J. De, (2008b). The components of plant tissue culture media II: organic additions, osmotic and pH effects, and support systems. In: Plant Propagation by Tissue Culture. Springer Netherlands, Dordrecht, pp. 115–173.

Gottschalk, U., (2009). Disposables in downstream processing. Springer, Berlin, Heidelberg, pp. 171–183.

Gupta, P.L., Lee, S.-M., Choi, H.-J., (2015). A mini review: photobioreactors for large scale algal cultivation. World Journal of Microbiology and Biotechnology, Vol. 31, pp. 1409–1417.

Häkkinen, S.T., Reuter, L., Nuorti, N., Joensuu, J.J., Rischer, H., Ritala, A., (2018). Tobacco BY-2 media component optimization for a cost-efficient recombinant protein production. Frontiers in Plant Science, Vol. 9, pp. 45.

Han, J., Zhong, J.-J., (2003). Effects of oxygen partial pressure on cell growth and ginsenoside and polysaccharide production in high density cell cultures of *Panax notoginseng*. Enzyme and Microbial Technology, Vol. 32, pp. 498–503.

Hellwig, S., Drossard, J., Twyman, R.M., Fischer, R., (2004). Plant cell cultures for the production of recombinant proteins. Nature Biotechnology, Vol. 22, pp. 1415–1422.

Helsieni, (2018). Helsieni – Sienet Helsingistä [WWW Document]. URL https://www.helsieni.fi/fi/etusivu/ (accessed 7.12.18).

Holland, T., Sack, M., Rademacher, T., Schmale, K., Altmann, F., Stadlmann, J., Fischer, R., Hellwig, S., (2010). Optimal nitrogen supply as a key to increased and sustained production of a monoclonal full-size antibody in BY-2 suspension culture. Biotechnology and Bioengineering, Vol. 107, pp. 278–289.

Holopainen, J.K., Kivimäenpää, M., Julkunen-Tiitto, R., (2018). New light for phytochemicals. Trends in biotechnology, Vol. 36, pp. 7–10.

Huang, T.-K., McDonald, K.A., (2009). Bioreactor engineering for recombinant protein production in plant cell suspension cultures. Biochemical Engineering Journal, Vol. 45, pp. 168–184.

Infors, (2018). INFORS HT - Shaker FAQ [WWW Document]. URL http://www.infors-ht.com/index.php/en/science-center/faq-en/987-shaker-faq (accessed 8.13.18).

Jeong, C.-S., Chakrabarty, D., Hahn, E.-J., Lee, H.-L., Paek, K.-Y., (2006). Effects of oxygen, carbon dioxide and ethylene on growth and bioactive compound production in bioreactor culture of ginseng adventitious roots. Biochemical Engineering Journal, Vol. 27, pp. 252–263.

Junne, S., Neubauer, P., (2018). How scalable and suitable are single-use bioreactors? Current Opinion in Biotechnology, Vol. 53, pp. 240–247.

Khanahmadi, M., Paek, K.Y., (2017). Bioreactor technology for sustainable production of valuable plant metabolites: challenges and advances. In: Crop Improvement. Springer International Publishing, Cham, pp. 169–189.

Kieran, P.., MacLoughlin, P.., Malone, D.., (1997). Plant cell suspension cultures: some engineering considerations. Journal of Biotechnology, Vol. 59, pp. 39–52.

Klöckner, W., Lattermann, C., Pursche, F., Büchs, J., Werner, S., Eibl, D., (2014). Time efficient way to calculate oxygen transfer areas and power input in cylindrical disposable shaken bioreactors. Biotechnology Progress, Vol. 30, pp. 1441–1456.

Kobayashi, Y., Akita, M., Sakamoto, K., Liu, H., Shigeoka, T., Koyano, T., Kawamura, M., Furuya, T., (1993). Large-scale production of anthocyanin by *Aralia cordata* cell suspension cultures. Applied Microbiology and Biotechnology, Vol. 40, pp. 215–218.

Krikorian, A.D., Berquam, D.L., (1969). Plant cell and tissue cultures: The role of Haberlandt. The Botanical Review, Vol. 35, pp. 59–67.

Kuhner, (2018). Home - shaker [WWW Document]. URL https://www.kuhner.com/en/home.html (accessed 6.26.18).

Kurata, H., Mochizuki, A., Okuda, N., Seki, M., Furusaki, S., (2000). Intermittent light irradiation with second- or hour-scale periods controls anthocyanin production by strawberry cells. Enzyme and Microbial Technology, Vol. 26, pp. 621–629.

Lee, C.W.T., Shuler, M.L., (2000). The effect of inoculum density and conditioned medium on the production of ajmalicine and catharanthine from immobilized *Catharanthus roseus* cells. Biotechnology and Bioengineering, Vol. 67, pp. 61–71.

Lee, J., Finn, C.E., (2012). Lingonberry (*Vaccinium vitis-idaea L.*) grown in the Pacific Northwest of North America: Anthocyanin and free amino acid composition. Journal of Functional Foods, Vol. 4, pp. 213–218.

Lehmann, N., Dittler, I., Lämsä, M., Ritala, A., Rischer, H., Eibl, D., Oksman-Caldentey, K.-M., Eibl, R., (2014). Disposable bioreactors for cultivation of plant cell cultures. In: Production of Biomass and Bioactive Compounds Using Bioreactor Technology. Springer Netherlands, Dordrecht, pp. 17–46.

Li, D., Wang, P., Luo, Y., Zhao, M., Chen, F., (2017). Health benefits of anthocyanins and molecular mechanisms: Update from recent decade. Critical Reviews in Food Science and Nutrition, Vol. 57, pp. 1729–1741.

Li, R., Wang, P., Guo, Q., Wang, Z., (2011). Anthocyanin composition and content of the *Vaccinium uliginosum* berry. Food Chemistry, Vol. 125, pp. 116–120.

Lloyd, G., McCown, B., (1980). Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Commercially-feasible micropropagation of mountain laurel, Kalmia latifolia, by use of shoot-tip culture., Vol. 30, pp. 421–427.

Mekonnen, M.M., Hoekstra, A.Y., (2010). Value of Water Research Report Series No. 48 Value of Water.

Merck Millipore, (2018). Mobius® Single-use Bioreactors | Single-use Manufacturing | Biopharmaceutical Manufacturing | Merck [WWW Document]. URL http://www.merckmillipore.com/FI/en/Mobius-Single-Use-Manufacturing/Mobius-Single-Use-Bioreactors/N76b.qB.fW0AAAFZmkxiYtcY,nav (accessed 6.26.18).

Meyer, J.., Pépin, M.-F., Smith, M.A.., (2002). Anthocyanin production from *Vaccinium pahalae*: limitations of the physical microenvironment. Journal of Biotechnology, Vol. 93, pp. 45–57.

Mokuolu, S., (2018). New standards define single-use materials qualification. Pharmaceutical Technology, Vol. 42, pp. 52–53.

Murashige, T., Skoog, F., (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia Plantarum, Vol. 15, pp. 473–497.

Murthy, H.N., Lee, E.-J., Paek, K.-Y., (2014). Production of secondary metabolites from cell and organ cultures: strategies and approaches for biomass improvement and metabolite accumulation. Plant Cell, Tissue and Organ Culture (PCTOC), Vol. 118, pp. 1–16.

Newman, L., Nixon, D., (2014). Farming in an agriburban ecovillage development. SAGE Open, Vol. 4, pp. 215824401456238.

Nohynek, L., Bailey, M., Tähtiharju, J., Seppänen-Laakso, T., Rischer, H., Oksman-Caldentey, K.-M., Puupponen-Pimiä, R., (2014). Cloudberry (*Rubus chamaemorus*) cell culture with bioactive substances: Establishment and mass propagation for industrial use. Engineering in Life Sciences, Vol. 14, pp. 667–675.

Nordlund, E., Lille, M., Silventoinen, P., Nygren, H., Seppänen-Laakso, T., Mikkelson, A., Aura, A.-M., Heiniö, R.-L., Nohynek, L., Puupponen-Pimiä, R., Rischer, H., (2018). Plant cells as food – A concept taking shape. Food Research International, Vol. 107, pp. 297–305.

Nosov, A.M., (2012). Application of cell technologies for production of plant-derived bioactive substances of plant origin. Applied Biochemistry and Microbiology, Vol. 48, pp. 609–624.

NUTRA, (2013). Diana Plant Sciences debuts cocoa ingredient derived from plant stem cells [WWW Document]. URL https://www.nutraingredients-usa.com/Article/2013/03/25/Diana-Plant-Sciences-debuts-cocoa-ingredient-derived-from-plant-stem-cells (accessed 7.12.18).

Ochoa-Villarreal, M., Howat, S., Hong, S., Jang, M.O., Jin, Y.-W., Lee, E.-K., Loake, G.J., (2016). Plant cell culture strategies for the production of natural products. BMB reports, Vol. 49, pp. 149–58.

Oksman-Caldentey, K.-M., Barz, W. (Wolfgang), (2002). Plant biotechnology and transgenic plants. M. Dekker.

Oplatowska-Stachowiak, M., Elliott, C.T., (2017). Food colors: Existing and emerging food safety concerns. Critical Reviews in Food Science and Nutrition, Vol. 57, pp. 524–548.

Pilarek, M., Sobieszuk, P., Wierzchowski, K., Dąbkowska, K., (2018). Impact of operating parameters on values of a volumetric mass transfer coefficient in a single-use bioreactor with wave-induced agitation. Chemical Engineering Research and Design, Vol. 136, pp. 1–10.

Poore, J., Nemecek, T., (2018). Reducing food's environmental impacts through producers and consumers. Science (New York, N.Y.), Vol. 360, pp. 987–992.

Ramachandra Rao, S., Ravishankar, G.., (2002). Plant cell cultures: Chemical factories of secondary metabolites. Biotechnology Advances, Vol. 20, pp. 101–153.

Rasche, S., Herwartz, D., Schuster, F., Jablonka, N., Weber, A., Fischer, R., Schillberg, S., (2016). More for less: Improving the biomass yield of a pear cell suspension culture by design of experiments. Scientific Reports, Vol. 6, pp. 23371.

Räty, N., (2017). Home bioreactor –Local food from plant cell cultures. Aalto university.

Raven, N., Schillberg, S., Kirchhoff, J., Brändli, J., Imseng, N., Eibl, R., (2011). Growth of BY-2 suspension cells and plantibody production in single-use bioreactors. In: Single-Use Technology in Biopharmaceutical Manufacture. John Wiley & Sons, Inc., Hoboken, NJ, USA, pp. 251–261.

Rea, G., Antonacci, A., Lambreva, M., Pastorelli, S., Tibuzzi, A., Ferrari, S., Fischer, D., Johanningmeier, U., Oleszek, W., Doroszewska, T., Rizzo, A.M., Berselli, P.V.R., Berra, B., Bertoli, A., Pistelli, L., Ruffoni, B., Calas-Blanchard, C., Marty, J.L., Litescu, S.C., Diaconu, M., Touloupakis, E., Ghanotakis, D., Giardi, M.T., (2011). Integrated plant biotechnologies applied to safer and healthier food production: The Nutra-Snack manufacturing chain. Trends in Food Science & Technology, Vol. 22, pp. 353–366.

Reuter, L.J., Bailey, M.J., Joensuu, J.J., Ritala, A., (2014). Scale-up of hydrophobin-assisted recombinant protein production in tobacco BY-2 suspension cells. Plant Biotechnology Journal, Vol. 12, pp. 402–410.

Sajc, L., Grubisic, D., Vunjak-Novakovic, G., (2000). Bioreactors for plant engineering: an outlook for further research. Biochemical Engineering Journal, Vol. 4, pp. 89–99.

Santos, R.B., Abranches, R., Fischer, R., Sack, M., Holland, T., (2016). Putting the spotlight back on plant suspension cultures. Frontiers in Plant Science, Vol. 7, pp. 297.

Santos, R.B., Pires, A.S., Abranches, R., (2017). Addition of a histone deacetylase inhibitor increases recombinant protein expression in *Medicago truncatula* cell cultures. Scientific Reports, Vol. 7, pp. 16756.

Sartorius, (2018). BIOSTAT® RM - Wave-Mixed Bioreactor | Sartorius [WWW Document]. URL https://www.sartorius.com/sartorius/en/EUR/products/bioreactors-fermentors/single-use/biostat-rm (accessed 6.26.18).

Sato, K., Nakayama, M., Shigeta, J., (1996). Culturing conditions affecting the production of anthocyanin in suspended cell cultures of strawberry. Plant Science, Vol. 113, pp. 91–98.

Schlatmann, J.E., Vinke, J.L., ten Hoopen, H.J.G., Heijnen, J.J., (1995). Relation between dissolved oxygen concentration and ajmalicine production rate in high-density cultures of *Catharanthus roseus*. Biotechnology and Bioengineering, Vol. 45, pp. 435–439.

Schmitz, C., Fritsch, L., Fischer, R., Schillberg, S., Rasche, S., (2016). Statistical experimental designs for the production of secondary metabolites in plant cell suspension cultures. Biotechnology Letters, Vol. 38, pp. 2007–2014.

Scragg, A.H., (2007). The production of flavours by plant cell cultures. In: Flavours and Fragrances. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 599–614.

Sen, R., Swaminathan, T., (2004). Response surface modeling and optimization to elucidate and analyze the effects of inoculum age and size on surfactin production. Biochemical Engineering Journal, Vol. 21, pp. 141–148.

Shaikh, S., Shriram, V., Kumar, T.K. and V., (2018). Establishment of callus and cell suspension cultures of helicteres isora L. Research in Plant Biology, pp. 01–07.

Shukla, A.A., Gottschalk, U., (2013). Single-use disposable technologies for biopharmaceutical manufacturing. Trends in Biotechnology, Vol. 31, pp. 147–154.

Su, W.W., (2006). Bioreactor engineering for recombinant protein production using plant cell suspension culture. In: Plan Tissue Culture Engineering. Springer Netherlands, Dordrecht, pp. 135–159.

Symrise, (2018). Company - Symrise AG [WWW Document]. URL https://www.symrise.com/company/ (accessed 7.12.18).

Tekoah, Y., Shulman, A., Kizhner, T., Ruderfer, I., Fux, L., Nataf, Y., Bartfeld, D., Ariel, T., Gingis-Velitski, S., Hanania, U., Shaaltiel, Y., (2015). Large-scale production of pharmaceutical proteins in plant cell culture-the Protalix experience. Plant Biotechnology Journal, Vol. 13, pp. 1199–1208.

Thanh, N.T., Murthy, H.N., Paek, K.Y., (2014). Optimization of ginseng cell culture in airlift bioreactors and developing the large-scale production system. Industrial Crops and Products, Vol. 60, pp. 343–348.

Vasilev, N., Grömping, U., Lipperts, A., Raven, N., Fischer, R., Schillberg, S., (2013). Optimization of BY-2 cell suspension culture medium for the production of a human antibody using a combination of fractional factorial designs and the response surface method. Plant Biotechnology Journal, Vol. 11, pp. 867–874.

Villegas-Velásquez, S., Martínez-Mira, A.D., Hoyos, R., Rojano, B., Orozco-Sánchez, F., (2017). Hydrodynamic stress and limonoid production in *Azadirachta indica* cell culture. Biochemical Engineering Journal, Vol. 122, pp. 75–84.

Werner, S., Eibl, R., Lettenbauer, C., Röll, M., Eibl, D., De Jesus, M., Zhang, X., Stettler, M., Tissot, S., Bürkie, C., Broccard, G., Kühner, M., Tanner, R., Baldi, L., Hacker, D., Wurm, F.M., (2010). Innovative, non-stirred bioreactors in scales from milliliters up to 1000 liters for suspension cultures of cells using disposable bags and containers – s swiss contribution. CHIMIA International Journal for Chemistry, Vol. 64, pp. 819–823.

Werner, S., Greulich, J., Geipel, K., Steingroewer, J., Bley, T., Eibl, D., (2014). Mass propagation of *Helianthus annuus* suspension cells in orbitally shaken bioreactors: Improved growth rate in single-use bag bioreactors. Engineering in Life Sciences, Vol. 14, pp. 676–684.

Wilken, S., Schuurmans, J.M., Matthijs, H.C.P., (2014). Do mixotrophs grow as photoheterotrophs? Photophysiological acclimation of the chrysophyte Ochromonas danica after feeding. New Phytologist, Vol. 204, pp. 882–889.

Wilson, S.A., Roberts, S.C., (2012). Recent advances towards development and commercialization of plant cell culture processes for the synthesis of biomolecules. Plant biotechnology journal, Vol. 10, pp. 249–68.

Xu, J., Zhang, N., (2014). On the way to commercializing plant cell culture platform for biopharmaceuticals: present status and prospect. Pharmaceutical bioprocessing, Vol. 2, pp. 499–518.

Yoshida, T., Lee, S.Y., Nielsen, J., Stephanopoulos, G., (2017). Applied bioengineering : innovations and future directions. John Wiley & Sons, Incorporated.

Zhang, X., Stettler, M., Sanctis, D., Perrone, M., Parolini, N., Discacciati, M., Jesus, M., Hacker, D., Quarteroni, A., Wurm, F., (2009). Use of orbital shaken disposable bioreactors for mammalian cell cultures from the milliliter-scale to the 1,000-liter scale. Springer, Berlin, Heidelberg, pp. 33–53.

APPENDIX A: CHEMICAL COMPOSITION OF MS MEDIUM -POWDER

Micro Elements	mg/l	µM
$CoCl_2.6H_2O$	0.025	0.11
$CuSO_4.5H_2O$	0.025	0.10
FeNaEDTA	36.70	100.00
H_3BO_3	6.20	100.27
KI	0.83	5.00
$MnSO_4.H_2O$	16.90	100.00
$Na_2MoO_4.2H_2O$	0.25	1.03
$ZnSO_4.7H_2O$	8.60	29.91

Macro Elements	mg/l	mM
CaCl2	332.02	2.99
KH2PO4	170.00	1.25
KNO3	1900.00	18.79
MgSO4	180.54	1.50
NH4NO3	1650.00	20.61

(Duchefa Biochemie, 2018b)

APPENDIX B: CHEMICAL COMPOSITION OF MCCOWN WOODY PLANT INCLUDING VITAMINS -POWDER

Micro Elements	mg/l	µM
$CuSO_4.5H_2O$	0.25	1.00
FeNaEDTA	36.70	100.00
H_3BO_3	6.20	100.27
$MnSO_4.H_2O$	22.30	131.94
$Na_2MoO_4.2H_2O$	0.25	1.03
$ZnSO_4.7H_2O$	8.60	29.91

Macro Elements	mg/l	mM
$CaCl_2$	72.50	0.65
$Ca(NO_3)2\ .4H_2O$	471.26	2.35
KH_2PO_4	170.00	1.25
K_2SO_4	990.00	5.68
$MgSO_4$	180.54	1.50
NH_4NO_3	400.00	5.00

Vitamins	mg/l	µM
Glycine	2.00	26.64
myo-Inositol	100.00	554.94
Nicotinic acid	0.50	4.06
Pyridoxine HCl	0.50	2.43
Thiamine HCl	1.00	2.96

(Duchefa Biochemie, 2018a)

APPENDIX C: VIABILITY DATA

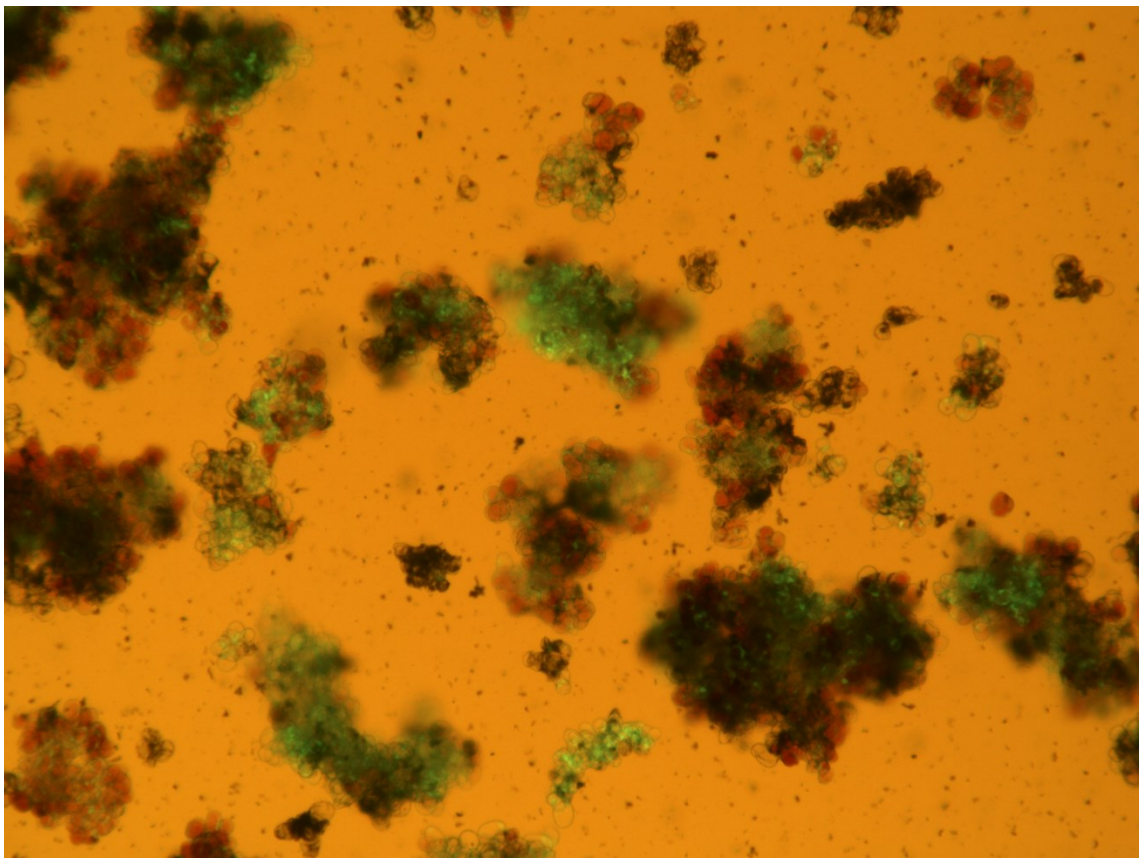

Figure 30. *Viability of culture Lingonberry-6 after 6 d cultivation by using FDA coloring and light microscopying. Black cells indicate about dead cells, light green color living ones. Small black background trash is from lysed cells.*