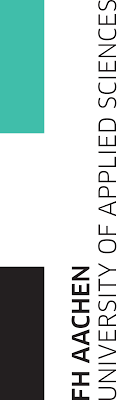
**\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

Fachhochschule Aachen, Campus Jülich

Fachbereich 03 Chemie und Biotechnologie

Studiengang: B.Sc.Biotechnologie

**\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

**Bachelorarbeit**

Culture optimization and characterization of three Porphyridium strains

for valuable compound production

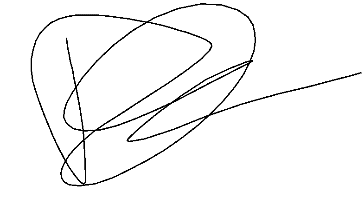
**Vorgelegt von**

**David Große**

**Matrikelnr.: 3142496**

Jülich, August 2022

Diese Arbeit ist von mir selbstständig angefertigt und verfasst. Es sind keine anderen als die angegeben Quellen und Hilfsmittel benutzt worden

****

\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

David Große

Diese Arbeit wurde betreut von:

1. **Prüfer Prof. Dr. Ingar Janzik**
2. **Prüfer Dr. Diana Reinecke-Levi**

# Danksagung

Ich möchte mich bei all denen bedanken, die mich bei diesem Bachelorprojekt unterstützt haben. Zunächst möchte ich Dr. Holger Klose für die Chance danken, dieses Projekt in der Alternative Biomasse Gruppe des IBG-2 am Forschungszentrum Jülich durchführen zu können. Prof. Dr. Ingar Janzik möchte ich für die Betreuung, sowie für die Erstkorrektur meiner Arbeit danken. Ein besonderes Lob und viel Dankbarkeit möchte ich Dr. Diana Reinecke-Levi für ihre ausgezeichnete Betreuung und tatenkräftige Unterstützung zusprechen. Darüber hinaus möchte ich mich bei Xinyu Gan für ihre Hilfe bei statistischen Fragen bedanken. Abschließend möchte ich mich bei Marion Roeb, Isabel Meuser, Andrea Neuwohner sowie Sophie Weber bedanken an die mich bei Fragen rund um das Labor wenden konnte, was die Arbeit sehr angenehm gemacht hat.

# Abstract

The optimal conditions to maximize phycoerythrin- (PE) and exopolysaccharide (EPS) production in the three photoautotrophic red marine microalgal strains of *Porphyridium purpureum* (SAG 1380-1a/-1d) and *cruentum* (UTEX 161) were researched. Design of experiment software (DoE) was used in planning and evaluation. Dependencies were found between light intensity, nitrogen and magnesium concentrations and the PE and EPS productivities. The maximal PE productivity (4,96 µg mL-1d-1) was found in *P. purpureum* SAG 1380-1d. Contrasting, the highest EPS productivity (5,75 g l-1 d-1) was found in *P. purpureum* SAG 1380-1a. However, optimization efforts in EPS production remained low due to low dependencies under tested growth conditions. The optimal product inducing conditions for all strains were identified at ~40 µmol photon m-2 s-1 and 9-15 mM nitrogen. The optimal magnesium concentration ranged between 5, 38 and 45 mM for UTEX 161, SAG 1380-1a and -1d, respectively. The pilot scale cultivation under greenhouse conditions could not be finished, because of temperature-stress during the cultivation.

# Zusammenfassung

Ziel dieser Arbeit sind die optimalen Bedingungen für die maximale Phycoerythrin (PE) und Exopolysaccharid (EPS) Produktion durch die photoautotrophe rote Mikroalge *Porphyridium purpureum* (SAG 1380-1a/-1d) and *cruentum* (UTEX 161) zu erforschen. Eine Design of Experiment Software wurde genutzt, um die Beziehung zwischen Lichtintensität, Stickstoff- und Magnesiumkonzentrationen hinsichtlich ihrer PE und EPS Produktivität darzustellen. Die maximale PE Produktivität wurde bei *P. purpureum* SAG 1380-1d (4,96 µg mL-1d-1) Strang und die maximale EPS Produktivität bei *P. purpureum* SAG 1380-1a Strang (5,75 g l-1 d-1) gefunden. Wir konnten keine Optimierung der EPS Produktion durchführen, da die Korrelation niedrig ist. Die besten Bedingungen liegen bei ~40 µmol Photonen m-2 s-1 und 9-15 mM Stickstoffkonzentration für alle Stämme. Die Magnesium Konzentrationen schwanken zwischen 5, 38 und 45 mM für UTEX 161, SAG 1380-1a und 1-d. Die Kultivierung im großen Maßstab wurde ausgeführt, jedoch konnten vergleichende Experimente zwischen Labor- und Industriemaßstab aufgrund von Temperaturstress nicht durchgeführt werden.

Content

[Danksagung 3](#_Toc111641974)

[Abstract 4](#_Toc111641975)

[Zusammenfassung 5](#_Toc111641976)

[Abbreviations 7](#_Toc111641977)

[**1.** **Introduction** 7](#_Toc111641978)

[**2.** **Materials and Methods** 8](#_Toc111641979)

[**2.1** **Materials** 8](#_Toc111641980)

[2.1.1 Chemicals 8](#_Toc111641981)

[2.1.2 Devices 9](#_Toc111641982)

[**2.2** **Growth parameters & analytical methods** 9](#_Toc111641983)

[2.2.1 Culture conditions 9](#_Toc111641984)

[2.2.2 Optical density 9](#_Toc111641985)

[2.2.3 Dry weight 10](#_Toc111641986)

[2.2.4 Total Chlorophylls 10](#_Toc111641987)

[2.2.5 Total Carotenoids 10](#_Toc111641988)

[2.2.6 Phycobiliprotein 10](#_Toc111641989)

[2.2.7 Exopolysaccharide 11](#_Toc111641990)

[2.2.8 Calculation productivity 11](#_Toc111641991)

[**2.3** **Design of Experiment (DoE)** 12](#_Toc111641992)

[2.3.1 Selection and range of independent variables 12](#_Toc111641993)

[**3.** **Results** 13](#_Toc111641994)

[**3.1** **Growth rate & biomass productivity** 13](#_Toc111641995)

[**3.2** **Pigment content** 14](#_Toc111641996)

[**3.3** **Phycoerythrin** 15](#_Toc111641997)

[**3.4** **EPS** 16](#_Toc111641998)

[**3.5** **Optimization** 17](#_Toc111641999)

[**3.6** **Pilot Scale Cultivation** 18](#_Toc111642000)

[**4.** **Discussion** 19](#_Toc111642001)

[**References** 21](#_Toc111642002)

# Abbreviations

|  |  |
| --- | --- |
| µmax | Maximal growth rate |
| µ | Growth rate |
| ASW | Artificial sea water |
| DoE | Design of experiment |
| DW | Dry weight |
| EPS | Exopolysaccharide |
| Mg | Magnesium |
| Mg25 | Magnesium, 25 mM |
| N | Nitrogen |
| N5 | Nitrogen, 5mM |
| OD | Optical density |
| Pi | Productivity |
| PBP  PE  PC  AP | Phycobilliproteins  Phycoerythrin  Phycocyanin  Allophycocyanin |
| *P. p.* | *Porphyridium purpureum* |
| *P. c.* | *Porphyridium cruentum* |
| RT | Room temperature |
| tCar | Total Carotenoid |
| tChl | Total Chlorophyll |
| tN | Total Nitrogen |
| tPO4-P | Total ortho-Phosphate |
| K | Relative growth constant |

# **Introduction**

Microalgae of the genus Porphyridium belong to the larger phylum of photoautotrophic marine red algae, the Rhodophyta. Many of them are high-value algae, due to their high content of polyunsaturated fat acids (PUFAs), phycobiliproteins (PBP) and exopolysaccharides (EPS) [1,2]. Yet, compare to the Rhodophyta-typical leafy macroalgae, Porphyridium cells lack the ability to grow in multicellular phenotypes. Instead, cells remain single cellular cultures of globular to polygonal cells, ranging between 7-12 µm in diameter [3]. Thus, Porphyridium species can be grown in conventional microalgal photobioreactors and gained a high economic value in various market sectors [13]. PUFAs, EPS, PBP, carotenoids and the chlorophyll-a can be used as nutraceuticals, natural colorants or antioxidants in food, feed, and cosmetics [4]. The auto-fluorescence of the PBP Phycoerythin is utilized in fluorescent marker in research and diagnostic [5]. Commercial producers can be found in Israel, USA, and China [16, 17, 18].

However, persisting challenges and problems in the up-scaling and the down-stream processing require further research. A major problem remains the variable cellular composition, which causes a cost-intensive down streaming. For example, the EPS are harvested by energy intensive centrifugation or solvent intensive precipitation. Furthermore, continuous biorefinery of several products include more cost intensive processes. PBP and EPS yields can vary largely under suboptimal culture and climate conditions. Therefore, one possible solution could be the strain optimization for given culture conditions strain to increase productivity while reduce production costs.

In this bachelor thesis were studied the (i) effect and (ii) optimization of three independent variables for the EPS- and PBP-productivity in the three microalgal strains *Porphyridium pupureum* SAG1380-1a and -1d, and *Porphyridium cruentum* UTEX 161. All three strains were known for their commercial EPS and PBP production [12, 17].

DoE software was used for planning and evaluation of the experiments. The selection and range of the independent variables (A) light intensity (B), nitrogen and (C) magnesium concentration was based on literature review. Light intensity and quality are the most important growth factors for photosynthetic microalgae. Thus, the synthesis of phycobiliproteins and chlorophylls in Porphyridium are highly dependent on the available light [6]. According to the literature, Porphyridium prefers lower light intensities (A) of 300-500 µmol photon m-2 s-1 [6, 21]. Nitrogen (B) and magnesium (C) are essential ions in the photosynthetic pigments and other cell compounds [12]. The highest rate of photosynthesis and product formation are expected under elevated nitrogen and magnesium concentrations. Optimal concentrations are indicated at 5-15 mM nitrogen [7, 14] and 5-45 mM magnesium [8]. Following lab-scale optimization, the Porphyridium strains with the highest PBP- and EPS productivity will be tested at pilot scale under the local climatic conditions in North Rhine Westphalia.

# **Materials and Methods**

## **Materials**

### Chemicals

**Tab. 1.** Chemical composition of the standard growth medium artificial seawater (ASW)

|  |  |  |  |
| --- | --- | --- | --- |
| Chemical |  | g L-1 | medium |
| 1. NaCl |  | 27 g | 27 |
| 1. MgSO4 + 7 H2O |  | 6,6 g | 6,6 |
| 1. MgCl2 + 6 H2O |  | 5,6 g | 5,6 |
| 1. CaCl + 2 H2O |  | 1,5 g | 1,5 |
| 1. KNO3 |  | 1,0 g | 1,0 |
| 1. KH2PO4 |  | 0,07 g | 0,07 |
| 1. NaHCO3 |  | 0,04 g | 0,04 |
| 1. Chelated Iron stock solution   FeCl3 + 4 H2O  0,05 M EDTA |  | 240 g  14,6 g | 1 mL |
| 1. 1 M TRIS HCL, pH 7,6 |  | 121 g | 20 mL |
| 1. Trace metal stock solution   ZnCl2  H3BO3  CoCl2 + 6 H2O  CuCl2 + 2 H2O  MgCl2 + 4 H2O  (NH4)6 Mo7O24 + 4 H2O |  | 40 mg  600 mg  15 mg  40 mg  400 mg  370 mg | 1 mL |

|  |  |
| --- | --- |
| ≥99,5% Dimethylsulfoxid (DMSO) | 20 mM Acetate buffer |
| 30% (v/v) Methanol | 96% Ethanol |
| 0,9 M KOH solution | 3,7% HCl |

**Tab. 2. Other used chemicals**

### Devices

**Tab. 3.** Devices used in cultivation and analysis

|  |  |  |
| --- | --- | --- |
| Device | Name and Type | Producer |
| Centrifuge | RC 6+ Plus | Sorvall® |
| Spectrophotometer | D-5000 | Hach-Lange® |
| Balance | MS Semi-Micro Balances | Mettler Toledo® |
| Ultrasonic device | 75186 | Vibra cell® |
| Heat block | Rotilabo-Block-Heater H250 | Roth® |
| Incubator | Minitron Floor | Infors® |
| Orbital shaker | DOS-20L | ELMI® |

## **Growth parameters & analytical methods**

### Culture conditions

The microalgal stains *Porphyridium purpureum* (SAG 1380-1a and -1d) were ordered from the Algal Culture of the University of Goettingen, Germany. The strain *Porphyridium cruentum* (UTEX 161) was ordered from the University of Texas, USA. All three strains were cultivated in standard artificial seawater (ASW) medium of Jones (1963) [8], see 2.1.1. The cultures were established in Erlenmeyer flasks of 50 to 2000 mL and shacked at 170 rpm, 20°C and light intensity and cycle of 30-60 µE and 12:12 h, respectively, Fig. 1A. Cultures were kept in batch mode and diluted at most 1:5, once a week. During experiments, cultures were tested in modified ASW-medium at a light-path of 1 cm under the light intensities of 40 to 500 µmol photon m-2 s-1, Tab. 4. The nitrogen and magnesium concentrations were adjusted between 5-15 and 5-45 mM, respectively, Tab. 4. Cultures for biomass production were kept in 2000 mL Erlenmeyer flasks at 170 rpm, RT and diurnal 300-600 µmol photon m-2 s-1. Cultures for pilot scale were kept in sharp bags of 5 L, which are aerated with air, 20-30°C and diurnal 100-400 µmol photon m-2 s-1, see Fig. 1B.

**A**

**Fig. 1.** Cultivation systems in (A) laboratory and (B) greenhouse

**B**

**A**

### Optical density

Optical density (OD) was measured in a spectrophotometer (D5000, Hach-Lange®). 1 mL culture was sampled and measured at 750 nm. Samples were diluted, if the density exceeded OD750 = ≥0,4 [9]. The growth rate (1) and doubling time (2) was calculated as noted below:

(1)

### Dry weight

(2)

Dry weight (DW) was determined by filtering 5 mL of culture through pre-weight and rinsed disk-filter (GF/C TM, Whatman). Filters were rinsed with 5 mL deionized water, dried at 100°C for 1h and left to cool in an exicator, overnight. Filter with retained cells were weight again (MS Semi-Micro Balances, Mettler Toledo®) and the dried biomass weight calculated according to [9]:

### Total Chlorophylls

The total chlorophyll content (tChl) was determined according to Boussiba *et al.* (1999) [11]. 1-5 mL culture were sampled, centrifuged at 3500 rpm for 5 min and the supernatant is discarded. The cell pellet was extracted in 5 mL DMSO at 70°C for 5 min and centrifuged again. The absorption of the supernatant was measured at 665 nm (D5000, Hach-Lange®). The chlorophyll content was calculated as noted below [10]:

### Total Carotenoids

The total Carotenoid content (tCar) was determined according to Boussiba *et al.* (1999) [11]. 1-5 mL culture were sampled, centrifuged at 3500 rpm for 5 minutes and the supernatant was discarded. The pellet was treated with 5 mL of 0,9 M KOH in 30% (v/v) methanol for 5 min, centrifuged and the supernatant discarded again. 50 µl of acetic acid and 5 mL of DSMO were added and extracted at 70°C for 5 min. After centrifugation, the adsorption of the supernatant was measured at 490 nm (D5000, Hach-Lange®). The carotenoid content was calculated as noted below [10]:

### Phycobiliprotein

The Phycobiliproteins (PBP) content were determined according to Kathiresan *et al.* (2006). 10 mL culture were centrifugalized at 3500 rpm for 5 min. The supernatant was discarded, and the cell pellet was washed twice with 10 mL acetate buffer with followed centrifugation for 5 min and dried overnight. 15 mL of acetate buffer was added. The cells were disrupted with an ultrasonic device for 5 min at an amplitude of 100% and a pulse of 5:1. The sample was filled up with acetate buffer to the final volume of 50 mL. After the centrifugation at 3500 rpm for 5 minutes, the absorption of the supernatant was measured at 280, 565, 620 and 650 nm (D5000, Hach-Lange®). The calculation of the individual pigment Phycocyanin (PC), Allophycocyanin (AC), and Phycoerythin (PE) can be seen below [11]:

### Exopolysaccharides

The exopolysaccharide (EPS) yield was determined gravimetrically according to an intern work instruction [10]. An unloaded tube was weighed [m1], filled with 50 mL culture and centrifuged at 3500 rpm for 5 minutes. The pellet was discarded, and the volume of the supernatant noted [V]. 150 mL of pure ethanol were subsequently added and slowly blended, until the EPS precipitated. The suspension was centrifuged at 3500 rpm for 5 minutes, the supernatant discarded, and the EPS-pellet dried at 40°C overnight. After drying, the tube was weighed again [m2]. The calculation for EPS yield can be seen below:

### Calculation productivity

The productivity of biomass, -tChl, -tCar, -PBP and -EPS were calculated as noted below:

## **Design of Experiment (DoE)**

### Selection and range of independent variables

|  |  |  |  |
| --- | --- | --- | --- |
| Run | A: Light [µmol photon m-2 s-1] | B: N [mM] | C: Mg [mM] |
| 1 | 40 | 5 | 25 |
| 2 | 40 | 10 | 5 |
| 3 | 40 | 10 | 45 |
| 4 | 40 | 15 | 25 |
| 5 | 270 | 5 | 5 |
| 6 | 270 | 5 | 45 |
| 7 | 270 | 10 | 25 |
| 8 | 270 | 10 | 25 |
| 9 | 270 | 10 | 25 |
| 10 | 270 | 15 | 5 |
| 11 | 270 | 15 | 45 |
| 12 | 500 | 5 | 25 |
| 13 | 500 | 10 | 5 |
| 14 | 500 | 10 | 45 |
| 15 | 500 | 15 | 25 |

The range of the variables (A) light intensities, (B) nitrogen and (C) magnesium concentration were chosen according to the literature. The light intensity ranged between 40-500 µmol photon m-2 s-1 [6, 21], the nitrogen concentration between 5-15 mM [7, 14] and magnesium concentration between 5-45 mM [8]. The experimental planning and analysis were supported by the DoE Software, Design-Expert 13®. The calculated number of experimental combinations (33 = 27 experiments) was reduced (15 experiments) by removal of redundant combinations. Table 4 shows the chosen experimental combinations by DoE.

**Tab. 4.** Values and combinations of independent variables

Four media were prepared for experiments 1-4 and 12-15, and 5 more media were prepared for the experiments 5-11, Tab. 4. An example for a calculation of the required KNO3 can be found below:

The same applied to the calculation of the MgSO4 + 7H2O admixture.

Experiments were conducted in biological triplicates and batch-cultures of 7 days. The dependent variables (OD, DW, PBP, EPS, tChl, tCar, tPO4-P, tN) were measured at the start and end of each experiment. These data were used for identifying correlations and optima of variables by the DoE Software, see Section 3.3 to 3.6.

Furthermore, the OD was measured throughout to characterize the growth behavior, Fig. 2.

# **Results**

As described in chapter 2.3.1, the experimental data were via DoE software to describe the correlations (R2) between the independent (A, B, C) and dependent variables such as biomass productivity, and PBP and EPS yields.

## **Growth rate & Biomass productivity**

**A**

Optical densities were measured to monitor growth and calculate growth rate (µ). The highest growth rate (0,09 d-1) and doubling time was found in the *P. p.* SAG 1380-1a under 270 µmol photon m-2 s-1 at N10 and Mg25, Fig 2A and Tab. 5. All strains showed a lag-phase in the first two days, Fig. 2A-C. Then the growth recovered from day 2 to 8, Fig. 2A-C. A slight decrease in growth was found in *P. p.* SAG 1380-1a between day 6-8, Fig. 2A. The variation of the data points was in acceptable range. The highest biomass productivity was found in strain UTEX 161 (0,63 g L-1 d-1), Tab 6.

**B**

The uptake of tN and tPO4-P from the medium were monitored and used to calculate the specific uptake rate for each strain under the tested growth conditions. The highest uptake rate for tPo4-P was found in *P. p.* SAG 1380-1a under 270 µmol photon m-2 s-1 at N10 and Mg25, Tab. 5. The highest N-uptake rate was found for the same conditions in *P. p.* SAG-1380-1d, Tab. 5.

**C**

**Fig. 2.** Optical densities over time at three light intensities in (A) *P. p.* SAG 1380-1a, (B) *P. p.* SAG 1380-1d and (C) *P. c.* UTEX 161. Light intensity: ⚪, 40 µmol photon m-2 s-1, ⚪, 270 µmol photon m-2 s-1, ⚪, 500 µmol photon m-2 s-1,  average optical density

**Tab. 5.** Results of three Porphyridium strains concerning of growth parameters and nutrients remove

|  |  |  |  |
| --- | --- | --- | --- |
|  | SAG 1380-1a | SAG 1380-1d | UTEX 161 |
| Pi Biomass [mg mL-1 d-1] | 0,60 | 0,62 | 0,63 |
| µmax[d-1] | 0,09 | 0,07 | 0,08 |
| Double time [d] | 7,6 | 9,6 | 8,6 |
| Remove tPo4-P [mg L-1 d-1] | 0,47 | 0,41 | 0,45 |
| Remove tN [mg L-1 d-1] | 0,28 | 1,43 | 0,57 |

## 

## **Pigment content**

Pigment contents were measured and compared for all experimental combinations. In all strains were pigment productivity and light intensity inversely correlated, Fig 3 A-C. Thus, the highest Phycoerythin (PE = 4,96 µg mL-1d-1) and phycocyanin (PC = 1,80 µg mL-1d-1) productivity was yielded in the strain *P. p.* SAG 1380-1d under the low light intensity of 40 µmol photon m-2 s-1 and N15 and Mg25, Fig 3B. The highest Allophycocyanin (AC = 3,48 µg mL-1d-1) productivity was identified in UTEX 161 under 270 µmol photon m-2 s-1, N10 and Mg25. In contrast, the AC productivity remained relative constant in SAG 1380-1a, Fig. 3A. The Pi decreased by xx% and turned negative under 270 and 500 µmol photon m-2 s-1, respectively. The decreases of AC Pi were xx% in SAG 1380-1d and UTEX 161 under 500 µmol photon m-2 s-1.

The highest tChl Pi was found in SAG 1380-1a (0,54 µg mL-1d-1) under 40 µmol photon m-2 s-1, N15 and Mg25, Fig. 3A. The highest tCar Pi was identified in strain SAG 1380-1a (0,18 µg mL-1d-1) under 270 µmol photon m-2 s-1 N15 and Mg 45, Fig. 3A. The Pi of tChl and tCar showed slight fluctuations between 0,09-0,54 µg mL-1d-1, Fig. 3A-C and no dependence to light, N and Mg. These results illustrate again, that light intensity is the most important variable in pigment production.

**C**

**Fig. 3.** Comparison of productivities for pigments synthesis in (A) *P.p.* SAG1380-1a, (B) *P.p.* Sag 1380-1d and (C) *P.c.* UTEX 161. ●Chlorophyll ●Carotenoid ●Phycocyanin ●Allophycocyanin ●Phycoerythrin; N5 Mg 25= nitrate 5 mM & magnesium 25 mM; exp. 1-4 low light intensity (40 µmol photon m-2 s-1), exp. 5-11 middle light intensity (270 µmol photon m-2 s-1), exp. 12-15 high light intensity (500 µmol photon m-2 s-1).

**B**

**A**

## **Phycoerythrin productivity**

3D surface contour plots illustrate the dependency of PE-productivity of the light intensity and the nitrogen or magnesium concentration in three Porphyridium strains, Fig. 4. The highest PE-productivity (4,96 µg mL-1d-1) was identified at high nitrogen level but low light intensity in *P.p.* SAG 1380-1d, Fig. 4B. Both *P.p.* strains, SAG 1380-1a and -1d, showed a sharp maxima in PE productivity at 40 µmol photon m-2 s-1 and 15 mM N, Fig 4A & 4B. Contrasting, *P.c.* UTEX 161 showed an high PE productivity over a wide nitrogen range (5-15 mM N) with a maximum (4 µg …) at 15 mM N, Fig 4C. Similarly, PE productivity (3,23 µg mL-1d-1) was increased at 40 µmol photon m-2 s-1 (R2 = 0,96) of the mg ? level, Fig 4F. The strain *P.c.* UTEX 161 showed a broad maximum in PE productivity over a wide range of Mg-concentrations, Fig 4F. In strong contrast the strains *P.p.* SAG 1380-1a and 1-d showed moderate increase in PE productivity for all tested light intensities and mg concentration, Fig E-F. Light is most decisive variable for the PE productivity.

|  |  |  |
| --- | --- | --- |
| SAG 1380-1a | SAG 1380-1d | UTEX 161 |
| **A: Light intensity [µmol photon m-2 s-1]**  40 132 224 316 408 500  **B: N [mM]**  **A**  **PE Pi [µg ml-1d-1]**  0 1 2 3 4 5 6  5 7 9 11 13 15 | **A: Light intensity [µmol photon m-2 s-1]**  40 132 224 316 408 500  **B: N [mM]**  5 7 9 11 13 15  **B**  **PE Pi [µg ml-1d-1]**  0 1 2 3 4 5 6 | **A: Light intensity [µmol photon m-2 s-1]**  **F**  **B: N [mM]**  5 7 9 11 13 15  40 132 224 316 408 500  **C**  **PE Pi [µg ml-1d-1]**  0 1 2 3 4 |
| **A: Light intensity [µmol photon m-2 s-1]**  **B: Mg [mM]**  5 15 25 35 45  **D**  0 1 2 3 4 5 6  **PE Pi [µg ml-1d-1]**  40 132 224 316 408 500 | **A: Light intensity [µmol photon m-2 s-1]**  **B: Mg [mM]**  5 15 25 35 45  40 132 224 316 408 500  **E**  0 1 2 3 4 5 6  **PE Pi [µg ml-1d-1]** | **A: Light intensity [µmol photon m-2 s-1]**  **B: Mg [mM]**  5 15 25 35 45  40 132 224 316 408 500  **PE Pi [µg ml-1d-1]**  0 1 2 3 4 |

**Fig. 4.** 3D surface contour plots illustrating the dependency of Phycoerythrin productivity of light intensity and nitrogen (A, B, C) or magnesium (D, E, F) concentration. Porphyridium strains left, SAG 1380ß-1a; center, SAG 1380-1d; right UTEX 161. Axis labeling: X1, light intensity [µmol photon m-2 s-1]; X2, nitrogen concentration [mM]; X3, magnesium concentration [mM]; Y, PE Pi[µg ml-1d-1]

## **EPS productivity**

3D surface contour plots illustrate the dependency of EPS productivity from light intensity and nitrogen or magnesium concentration in three Porphyridium strains, Fig. 5. The highest EPS Pi (1,53 g L-1d-1) was identified in *P.p.* SAG 1380-1a at low nitrogen level and low light intensity, Fig 5A & B. R2 vary between 0,64-0,75. The mathematical model were modified by addition of a summand (K = 2 g L-1d-1), because of negative productivities. The strain *P.p.* SAG 1380-1a and -1d showed sharp maxima in EPS productivity at 40 µmol photon m-2 s-1 and 5 mM N, Fig. 5A & B. Contrasting, the productivity in UTEX 161 showed an increase over a wide nitrogen-range, Fig. 5C.

The highest EPS Pi (1,76 µg mL-1d-1) was identified in SAG1380-1a at low light intensity and 45 mM Mg, Fig 5D-F. The strain *P.p.* SAG 1380-1a and SAG1380-1d showed a sharp maxima in EPS Pi, Fig 5D. In strong contrast the strain *P.c.* UTEX 161 showed an increase in EPS Pi over wide magnesium range, 5F.

|  |  |  |
| --- | --- | --- |
| SAG 1380-1a | SAG 1380-1d | UTEX 161 |
| **B: N [mM]**  **A: Light intensity [µmol photon m-2 s-1]**  40 132 224 316 408 500  0 1 2 3 4  **EPS Pi [g L-1d-1]**  **A** | **B: N [mM]**  0 1 2 3 4  5 7 9 11 13 15  **EPS Pi [g L-1d-1]**  **B**  5 7 9 11 13 15  **A: Light intensity [µmol photon m-2 s-1]**  40 132 224 316 408 500 | **C**  **B: N [mM]**  **A: Light intensity [µmol photon m-2 s-1]**  0 0,5 1 1,5 2 2,5 3  5 7 9 11 13 15  40 132 224 316 408 500  **EPS Pi [g L-1d-1]** |
| 40 132 224 316 408 500  **A: Light intensity [µmol photon m-2 s-1]**  **B: Mg [mM]**  0 1 2 3 4  **D**  5 15 25 35 45  **EPS Pi [g L-1d-1]** | 40 132 224 316 408 500  **A: Light intensity [µmol photon m-2 s-1]**  **B: Mg [mM]**  0 1 2 3 4  **EPS Pi [g L-1d-1]**  **E**  5 15 25 35 45 | 40 132 224 316 408 500  **A: Light intensity [µmol photon m-2 s-1]**  **B: Mg [mM]**  0 0,5 1 1,5 2 2,5 3  **EPS Pi [g L-1d-1]**  **F**  5 15 25 35 45 |

## **Optimization**

**Fig. 5.** 3D surface contour plots illustrating the dependency of EPS productivity of light intensity and nitrogen (A, B, C) or magnesium (D, E, F) concentration. Porphyridium strains left, SAG 1380ß-1a; center, SAG 1380-1d; right UTEX 161. Axis labeling: X1, light intensity [µmol photon m-2 s-1]; X2, nitrogen concentration [mM]; X3, magnesium concentration [mM]; Y, EPS Pi[µg ml-1d-1]

|  |  |  |  |
| --- | --- | --- | --- |
|  | SAG 1380-1a | SAG 1380-1d | UTEX 161 |
| A: Light intensity [µmol photon m-2 s-1] | 40,01 | 40,74 | 40,01 |
| B: N [mM] | 13,92 | 8,98 | 15 |
| C: Mg [mM] | 38,29 | 44,99 | 5,01 |
| Calculated PE Pi [µg mL-1d-1] | 3,54 | 3,06 | **4,54** |
| Calculated EPS Pi [g l-1d-1] | **5,75** | 3,15 | 2,26 |

The optimization of three independent variables for maximization of PE and EPS Pi were calculated with the software Design Expert 13, see Tab. 6. The Optimum would be 40-41 µmol photon m-2 s-1, 9-15 mM nitrogen and for all strains. The magnesium concentration is rather low for UTEX 161 compared to SAG 1380-1a and -1d. The optimization showed that a high production of PE will be expect in *P.c.* UTEX 161 and EPS production in *P.p.* 1380-1a, see Tab. 6.

**Tab. 6.** Calculated optimization for maximization of PE and EPS productivity.

## **Pilot Scale Cultivation**

The planned pilot scale cultivation was partial successfully done. We up-scaled the cultivation volume in plastic bags to ~30 l for the Porphyridium strain SAG 1380-1d and UTEX 161, Fig. 6. However, sudden hot weather events lead to a sharp increase in temperature (~50 °C) and hindered the cultivation in the greenhouse. Adaptation measures were implemented, but the planned comparative experiments could not be finalized, due to the lack of time.

**Fig. 6.** Cultivation of *P. p.* SAG 1380-1d and *P. c.* UTEX 161 in plastic bags under greenhouse conditions.

# **Discussion**

In this study were three commercial Porphyridium strains tested for the coproduction of PE and EPS production under various growth conditions.

The OD-measurements were used to calculate the increase in growth. Overall, we found comparable growth pattern in all three Porphyridium strains. The µ ranged between 0,07- 0,09 (k = 0,25-0,31) and were half in comparison to the literature values (k = 0,76-0,84) [9]. This result was also confirmed by other literature [8, 14]. The authors used similar culture conditions (150 mL cultures in 250 mL Erlenmeyer flasks, ASW medium, shaker at 150-170 rpm and a “cool white” fluorescent lamp at 53-62 µmol photon m-2 s-1), but the serial number and quality of the lamp were not named. This might be due to growth limiting factors such as light illumination and spectrum. As a solution the light cycle might be increased to 14:10-18:6 for more photosynthesis. Furthermore, the duration of experiments should be longer than 7 days, because of cell acclimation [8, 9, 14].

Further, we confirmed that both, chlorophyll, and carotenoid content are no reliable growth indicators. Porphyridium gains most photosynthetic energy through the PBP [19]. The highest PE productivity was in *P.p.* SAG 1380-1d. We found a similar PE productivity and percent by weight (3,8 % PE) by comparison with literature (3,3 % PE), respectively [12]. The authors used similar growth conditions (100 mL culture in 250 mL Erlenmeyer flasks, ASW medium, shaker at 80 rpm, 18 µmol photon m-2 s-1, T = 25°C), so that the results are comparable.

We found an optimal and wide range of N and Mg in UTEX 161, so it seems that the strain reacts sensitive to N and Mg. We found literature, which showed evidence to our results [14]. The author used the same strain and similar culture conditions (UTEX 161, 150 mL culture in 250 mL Erlenmeyer flasks, f/2 medium, shaker at 150 rpm, 98 µmol photon m-2 s-1, T = 23°C) and they analyzed the effect of different N concentrations on growth and carbohydrate formation. We found that *P.c.* UTEX 161 metabolized low nitrogen as well as high concentrations, but low concentrations conditioned N limitation in Porphyridium [14] and a higher N concentration were preferred. Furthermore, we didn’t find comparable studies for Mg sensitivity, so that the result cannot be verified. We found the optimal point for N in SAG 1380-1a and -1d. For that reason, experiments should be done between light intensities 20-200 µmol photon m-2s-1, in *P.p.* SAG 1380-1a & -1d. Moreover, the SAG strains did not react to Mg, so it seems that Mg is not necessary for PE production in SAG 1380-1a and -1d. The hypothesis cannot be supported by literature, because of the same reason as above described.

For increasing the PBP Pi, we found an application of additional blue, fluorescent light (400-430 nm), which can increase the PBP content, by stimulation of PE [8, 15]. The technical practicability will be proved for our incubator.

We found no reliable optimum for EPS productivity, since correlations were low (R2 = 0,64-0,75). Porphyridium can use secreted EPS as a second energy source [22] for that reason we found negative EPS Pi.

The DoE software calculated the potential production of PE and EPS for all strains. The optimization for PE production shows clearly that the strain UTEX 161 produces mainly PE and PBP according to literature [12, 17]. Therefore, the PE production of UTEX 161 is gradable by comparison of our results. The optimization of EPS production was not evaluated, because of low correlations.

The total remove of tPo4-P was successfully done, because we found slightly less than the calculated tPo4-P and the results are similar. However, the measurement of N is not a reliable method, because we found twice as much of N at the beginning of an experiment as the calculated concentration. It is possible, that cell debris and contaminations cause this effect.

The greenhouse cultivation was partial successfully done because the cultures were killed by heat. Porphyridium is affected to heat and literatures confirm this issue [6, 20]. Industrial processes are also concerned [21], so that simple and cheap solutions is used. For low technical costs, Porphyridium should be cultivated seasonal in greenhouses in the spring or autumn in plastic bags at 15-25°C under 40-200 µmol photon m-2s-1 and aerated with air. The technical costs increase in winter and summer. In summer, the cultures should be cooled and shaded. During winter, cultures could be heated and illuminated by additional light sources.

The further outlook shows that we will use our optimized growth medium and the strains *P.p.* SAG 1380-1a and *P.c.* UTEX 161 for our greenhouse cultivation in the spring or autumn.

# **References**

1. Duboc, P., Mollet, B. (2001). Applications of exopolysaccharides in the dairy industry. International Dairy Journal, 11, 759–768.
2. Ates, O. (2015). Systems biology of microbial exopolysaccharides production. Frontiers in Bioengieneering and Biotechnology*, 3*, 1-16.
3. Drew, K., & Ross, R. (1965). Some generic names in the Bangiophycidae. Taxon, 14, 93-99.
4. Patel, A. K, Laroche, C., Marcati, A., Ursu, A. V., Jubeau, S., Marchal, L., Petit, E., Djelveh, G, Michaud, P. (2012). Separation and fractionation of exopolysaccharides from *Pohyridium cruentum*. Bioresource Technology, 145, 345-350.

Heath, O. (1972). Physiologie der Photosynthese. Thieme, 230-252

Dermoun, D., Chaumont, D. (1992). Modelling of growth of *Porphyridium cruentum* in connection with two interdependent factors: light and temperature. Bioresource Technology, 42, 113-117

Li, T. Xu, J., Wu, H., Jiang, P., Chen Z., Xiang W. (2019).: Growth and biochemical composition of *Porphyridium pupreum* SCS-02 under different nitrogen concentrations. Marine Drugs, 17, 1-14

Medina-Cabrera, E. V., Rühmanna B., Schmida, J., Sieber V. (2020): Optimization of growth and EPS production in two Porphyridium strains. Algal Research, 49, 6-22

1. Jones, R., Speer, H., Kury, W. (1963). Studies on the growth of the red alga Porphyridium cruentum. Physiologia Plantarum*,* 16, 43-636.
2. Drobietz, D. (2020). Quantifizierung von verschiedenen Zuckern mittels IC. Intern work instruction of the FZ-Juelich, 1-5
3. Boussiba, S., Bing, W., Yuan, J.-P., Zarka, A., Chen, F. (1999). Changes in pigments profile in the green alga Haematococcus pluvialis exposed to environmental stresses. Biotechnology Letters, 21, 601-604.
4. Kathiresan, S., Sarada R., Bhattacharya S., Ravishankar, G.A. (2006). Culture Media Optimization and Phycoerythrin Production from Porphyridium purpureum. Biotechnology Bioengineering, 96, 456-463.
5. Sircus, M. (2009). Magnesium: The Lamp of Life- Chlorophyll, DNA, DHEA and Cholesterol. Dr. Sircus. Retrieved from https://drsircus.com/magnesium/magnesium-the-lamp-of-life/
6. Razaghi, A., Godhe, A., Albers, E. (2012). Effects of nitrogen on growth and carbonhydrate formation in *Porphyridium cruentum*. Versita, 9, 156-162.
7. Adda, M., Merchuk, J. C., Arad, S. (1986). Effect of nitrate on growth and production of cell-wall Polysaccharide by unicellular red alga Porphyridium. Biomass, 10, 131-140
8. French Associates Institute for Agriculture and Biotechnology of Drylands  
   Jacob Blaustein Institute for Desert Research. Retrieved from https://in.bgu.ac.il/en/bidr/FAAB/Pages/cohen.aspx
9. UTEX Culture Collection of Algae at the University of Texas at Austin. UTEX 161 Porphyridium cruentum Retrieved from <https://utex.org/products/utex-0161?variant=30991236333658>
10. South China Sea Institute of Oceanology Retrieved from <http://english.scsio.cas.cn/gb2020/>
11. Fleurence, J. (2003). R-phycoerythrin from red macroalgae: strategies for extraction and potential application in biotechnology. Applied Biotechnology. Food Science and Policy, 1, 1-6
12. Guihèneuf, F. & Stengel D. B. (2015). Towards the biorefinery concept: Interaction of light, temperature and nitrogen for optimizing the co-production of high-value compounds in Porphyridium purpureum. Algal Research, 10, 153-163
13. Vonshak, A., Cohen, Z. & Richmond A. (1984). The Feasibility of Mass Cultivation of Porphyridium. Biomass, 8, 13-25
14. Mutmainnah, N., Risjani, Y., Hertika, A. (2018) Growth Rate and Chemical Composition of Secondary Metabolite Extracellular Polysaccharide (EPS) in Microalga Porphyridium cruentum. J. Exp. Life Sci., 8, 97-102