Biotechnological potential of an isolated and a known green microalgae strain and their cultivation in biofilm based reactors

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PhD Thesis

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Szeged

2016

Background

Microalgae are a very diverse and large group of photosynthetic microorganisms, including prokaryotic and eukaryotic members as well. They play a significant role in oxygen generation, CO_2 fixation and primary producers of organic matter thus they are inevitable parts of the food chain. Microalgae were observed in all sorts of habitats, including for instant deep sea regions, tropical areas and even under polar ice and snow; tolerating extreme pH, salinity, temperature etc., both in planktonic and benthonic forms. This diversity provides possibilities for a wide range of biotechnological applications, from food and feed industry, pharmaceutical production, to agriculture, energy generation from biofuels or via biogas application, moreover wastewater treatment and nutrient recovery.

In the last decades, the interest of researchers moved towards biodiesel production from the accumulated lipid of the microalgae biomass, as fossil fuels prices drastically increased and availability decreased, along with the recognition of the effects of industrial activity on climate change. Microalgae are not only suitable for biodiesel formation but thanks to their diversity, a wide range of other secondary metabolites with biotechnological importance are present, as well. For example EPA, DHA β-carotene and astaxanthin are among the most marketable compounds of the microalgae biomass as human nutraceuticals. However, real large scale, industrial production of microalgae based high value added compounds is restricted and often extremely costly. Some of the main bottlenecks are the low cell density in suspensions and thus high dewatering cost of biomass; additionally the natural characteristic of most microalgae, that enhanced lipid or astaxanthin accumulation only occur under growth limiting conditions, such as low temperature or nutrient limitation, which causing low productivity rates.

Current large scale, commercial microalgae cultivation technologies are exclusively based on suspension cultivation, which consequently coupled with high energy, labour and volume demanding downstream dewatering processes e.g. flocculation, centrifugation, filtration and sedimentation. The cost of concentrating the biomass from the low cell density medium (between 0.5-10 g L^{-1} dry weight) can reach up to 20-30% of the total production cost, hence reducing the competitiveness of microalgae based products. However, biofilm based cultivation techniques can overcome the existing problems of the suspension based methods. Among the beneficial characteristics of biofilm based PBRs, the immobilization of cells and thus their separation from water bodies are one of the major ones. Despite the numerous setup designs and constructions of biofilm-based photobioreactors, biomass density is reported in the range of 37-200 g dry weight kg^{-1} wet weight.

Several different biofilm design and set-up have been developed and tested with different algae species for different purposes, however the amount of available information is far below

the amount of data and knowledge of suspension cultivation, either of open or closed systems. Also many of those have not yet been optimized for large scale application yet, which results in lacking of actual efficiency values; and their comparison is often problematic.

Objectives

The present PhD research focuses on the comparison of two biofilm based cultivation methods, namely the Algadisk and the Twin Layer system for high value compound production. The biotechnological potential of isolated and known microalga strains and the performance of these selected algae in the biofilm based Algadisk and Twin Layer reactors are also monitored.

In order to conduct such a research, the following specific aims are determined:

1. Isolation and selection of a microalga strain that presents the required characteristics, namely surface attachment, fast reproduction and biomass production, high content of valuable metabolites, such as lipids. Isolation places are natural water basins in Central Europe.
2. The selected strain and other available, known microalgae species, *Chlorella sorokiniana* and *Haematococcus pluvialis* are examined for surface attachment abilities. To clarify some relations between surface material and cell attachment using numerous specifically designed surface materials with special surface coatings by polyelectrolytes in negative or positive charge.
3. The newly designed, laboratory scaled Algadisk reactor operates with the isolated strain, *Chlorella* sp #34 and its biomass and lipid production are monitored and compared under different parameters such as low and high irradiation; optimized culture medium and artificial fertilizer. Besides the biomass quantity and quality, it will be examined how stable the system is considering contamination, mechanical problems, whether it can operate continuously and how the biofilm formation is affected by the harvest and regrowth cycles.
4. The Twin Layer system is investigated regarding the effects of light intensity and application of stress from the culture medium on the *H. pluvialis* microalga for astaxanthin production which is a biotechnologically important compound.
5. Finally, the two biofilm based cultivation concepts are analyzed and compared to each other and to other published biofilm systems.

Materials and methods

1. After sampling, microalgae consortium were enriched in two rich liquid medium, Sueoka and A9 medium. Samples were platted onto agar plates of the same medium and with single cell separation, monocultures were established.
2. First step of selection of the isolated samples happened by setting up liquid cultures and their optical density at 550 nm wavelength were followed regularly for about 2 weeks with a spectrophotometer. Additionally, lipid accumulation of the strains was detected by staining the cells with the lipid selective Nile Red dye under a fluorescence microscope.
3. The best performing strain was selected and identified by molecular methods using 5.8S rRNA, 18S rRNA, 28S rRNA genes, and ITS1 and ITS2 genome sequences.
4. For primary attached cultivation studies, a special, closed cultivation system was designed in order to test several surface materials with combination of polyelectrolyte layers. Performance was evaluated by gravimetrically determining the highest biomass formation.
5. Biomass production of *Chlorella* sp #34 in the Algadisk reactor, cultivated on M8-a medium, was followed by scrapping off the biofilm from the disk surfaces with a metal scraper in variable time periods. Collected wet biomass was weighted, then oven dried and weighted again. The measured values were used to calculated productivity, biomass yield, biomass yield on light and density.
6. Total lipid content and FAME composition were analyzed by GC-MS, after extraction and methylation of the fatty acids present in the dry biomass.
7. Total nitrogen content of the medium was followed by TOC Combustion Analyzer after removing cells and other particles from the liquid by centrifugation.
8. In the Twin Layer system, biomass production of *H. pluvialis* was determined by removing the polycarbonate membranes from the system and freeze-drying them. The measured dry weight was then used for biomass yield, productivity and for biomass yield on light calculations.
9. The astaxanthin content of the *H. pluvialis* biofilm was analyzed by using DMSO on the freeze-dried biomass to extract astaxanthin, which was quantified by spectrophotometry. Astaxanthin accumulation was triggered by applying nitrogen free or additional NaCl salt contening (0.05%, 0.2% and 0.4%) BBM medium to the system.

Results

1. Due to the isolation method, chances were increased to collect biofilm forming microalgae, moreover higher cell number could be taken from the sites. Based on this method, 58 samples were collected, of which further in 158 monocultures were separated. However on longer terms, not all of the monocultures could be maintained, approximately 50% was discarded from the collection. During the growth determination, the 102 isolated samples expressed a wide range of productivity ranging from about 0.1 to 3.8 optical density. Some of the best performing isolates were the followings: OD_{550nm} > 3: A9-17a; A9-25b; A9-26; A9-44b; A9-45; $OD_{550nm} \sim 1.5$: SH-25a and SH-34. The Nile red dying allowed observing the presence of lipid in the cells. In some cases, the lipid was located in well-distinguishable droplets inside the cell, while in other samples the whole cell emitted yellow light. However this method it was not suitable for quantitative determination of lipids, thus GC-MS was used on certain samples. The values of total fatty acid content in dry weight basis are varying greatly from 0.8% to 14.9%. Highest value was reached in SH-34, 149 mg FAME g^{-1} DW, for this reason, SH-34 sample was selected to go further with biofilm growth experiments. Based on the molecular identification and alignment search in the available database, the SH-34 sample was identified as a strain belonging to the order *Chlorellales.*
2. The primary attachment experiments showed that in case of *Chlorella sp*. #34 algae the most striking difference can be seen between the surface charges. On the negatively charged materials, about double biomass yield was measured as on the positively charged or non-coated surfaces, reaching 2.2 g m^{-2} in 7 days on PS substrate with coating #3. Lower biomass production was achieved by the *C. sorokiniana* strain, the highest value; 1.2 g m^{-2} was measured on PET with coating #1, on a positively charged surface. *H. pluvialis* showed the highest biomass yield, 8.5 g m^{-2} on PET with coating #3. Overall, the biomass yield was about 3.5 times and 7 times increased compared to *Chlorella sp*. #34 and *C. sorokiniana* species. Clear connection could not be drawn concerning the relationship between cell attachment and surface characteristics, such as charge. However, some coatings and surface material could be excluded from further application. Coating #2 was very unstable; the polyelectrolyte layers were peeling off from the substrate.
3. The laboratory scaled Algadisk reactor, under low light intensity and with full M8-a medium, was running continuously for 98 days, and 7 growth-harvest cycles occurred in different time lengths. After the primary biofilm formation (Harvest #1), which required 18 days, due to the slow attachment of cells to disk surfaces and biofilm formation, biofilm was harvested in every 7-8 days. By this regular harvesting, the biomass production showed an increasing tendency between 2.28 g $(m^2d)^{-1}$ and 3.23 g $(m^2d)^{-1}$.The

average biomass yield is about 17 g DW surface m^{-2} during the regular harvests. Biomass density stabilized around 200 g DW kg^{-1} WW.

4. The second experiment of the lab-scale Algadisk reactor with increased light intensity and full M8-a medium presented here was operated over 43 days and 4 growth-harvest cycles occurred. Even though the biomass productivity increased from harvest to harvest, however it remained lower compared to the previous experiment, 1-1.5 g DW $(m^2day)^{-1}$. The average biomass density was also lower, it varied around 100 g DW kg^{-1} WW, which is about half of the values found in the previous experiment. Both tested disk material, PVC and PP showed similar results, however biofilm growing on PVC reached higher productivity, biomass yield and light use efficiency as well. The reasons behind reduced biomass formation are still unknown and require further investigation.
5. Using artificial fertilizer as growth medium at low light conditions provided a good substitution for the expensive and labor demanding standard media, productivity remained only slightly below the values reached in M8-a medium, nonetheless problems of pH regulation raised. Highest biomass productivity was calculated to be 1.7 g DW $(m^2day)^{-1}$ with 130 g kg^{-1} biomass density.
6. Total lipid concentration of biomass and total lipid productivity was analyzed in all experiments under low (Harvests #5, 6, 7) and high light intensities with both disks material, PVC and PP and also by using fertilizer as a medium after each harvesting point. Total fatty acid contents of the biomass grew on full media and fertilizer are low, around 4 (w/w) %, 5.5 (w/w) % and 6.5% at low, high light conditions, and with artificial fertilizer respectively. Fatty acid productivity, 100 mg FAME $(m^2day)^{-1}$ was highest at low light intensity, due to the high biomass produced. A specialized experiment was designed and carried out to increase the FAME content of the biofilm. In the two- step approach, the biofilm grew first on full media, then medium was changed to N free version. Nitrogen starvation had an impact on the lipid content in cells, the fatty acid content increased more than 2 times compared to the other conditions, reaching 9.5% of DW. However the accumulation process is very slow. Additionally, the regrowth of the harvested biofilm on full medium did not occur, which is a major drawback in the Algadisk system.
7. Regarding the quality of the fatty acids, the dominant ones were palmitic acid, heptadecenoic acid, oleic acid, linoleic acid and α-linolenic acid, displaying minor differences among the experiments. Biodiesel application could be possible based on the FAME compositions, as medium chain length, mono- and di-saturated fatty acids are presents in large ratio. Other valuable fatty acids such as omega-3 and omega-6 polyunsaturated fatty acids were detectable however, in negligible concentration. Due to

the low total lipid content, we could conclude that *Chlorella* sp #34 would not be suitable for biodiesel production.

8. Cultivation of *H. pluvialis* in a biofilm based technology was successful in the Twin Layer system. Attempts to grow this microalga in the lab-scale Algadisk reactor has failed, most probably due to settlement of cells. Due to biofilm cultivation of *H. pluvialis*, even stressing light intensities, such as 219 μmol photons $(m^2s)^{-1}$ did not limit growth, moreover it enhanced. Biomass yield in the Twin Layer reactor was strongly dependent on the applied light intensity, increasing from 31 g m^{-2} at 26 μmol photons $(m^2s)^{-1}$ to 109 g m^{-2} at 219 μmol photons $(m^2s)^{-1}$. The biomass growth was linear during the entire time of the 18 days of the experiment. Biomass productivity was calculated based on linear regression ($R^2 > 0.93$); reaching 5.8 g $(m^2\ d)^{-1}$ at 219 μmol photons $(m^2\ s)^{-1}$.
9. Astaxanthin production was induced by the combination of high light (219 μmol photons $(m^2s)^{-1}$) and by the N limitation, from about 0.5% to 3.5%. The other applied stresses, NaCl, did not change the astaxanthin content in the examined time period, remained below 1%. Even though stress factors were applied to the system on day 8, the biomass increased linearly during the whole cultivation period, resulting in values from 5.13 to 6.7 g $(m^2\ d)^{-1}$. This consequently resulted in high astaxanthin production rate under N limitation, 300 mg $(m^2\ d)^{-1}$. The value is comparable to other published data on astaxanthin production and asserts a marketable technology.
10. Summarizing the observations of the presented biofilm systems, the Algadisk technology is suitable for long term, continuous operation with regular growth-harvest cycles. During the operation, only minor technical issues occurred, including stop of disk rotation and pH regulation. Significant contamination was not observed either. The Twin Layer system also operated without problems, however the Algadisk technology could be still considered closer to large scale, continuous application than the previous one, due to the regular growth-harvesting cycles, the partly automatized harvesting method and the scalable, competitive disk material. After further optimization of growth conditions and high value added compound production, both of the cultivation technologies have great potential for efficient, large scale biomass production, thanks to the major benefits of biofilm based cultivation of microalgae, including high biomass density, better light utilization, reduced water consumption, increased footprint based production and ease of stress induction.

Scientific publications

*<u>**Sebestyén P**</u>, Blanken W, Bacsa I, Tóth G, Martin A, Bhaiji T, Dergez Á, Kesserű P, Koós Á, Kiss I (2016) **Upscale of a laboratory rotating disk biofilm reactor and evaluation of its performance over a half-year operation period in outdoor conditions.** *Algal Research*, Vol. 18, 266-272
IF: 5.014

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Sum of impact factors: 10.324

*- PhD dissertation based on this scientific publication

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