Growth optimization of *Parietochloris incisa* for outdoor biomass production in Vertical Tubular Photobioreactors

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Abstract

Considerable interest had been focused on the freshwater green microalga, *Parietochloris incisa*, due to its ability to accumulate substantial quantities of arachidonic acid (AA). The current source of AA is a fungus, *Mortierella alpina* and *P. incisa* is being acknowledged as a potential plant source of AA at a commercial scale. This work involved growth optimization for biomass production in outdoor conditions using a Vertical Tubular Photobioreactor. The effect of different media formulations and nitrogen concentrations on biomass productivity were studied and the optimal cell density was defined. It was observed that high concentrations of nitrogen and other nutrients did not support significantly higher productivities. Highest biomass productivity was obtained in Bold Basal medium with 1.5 gl⁻¹ sodium nitrate at cell density range of 4.65 and 3.32 gl⁻¹ for laboratory and outdoor grown inocula, respectively. The harvesting cycle of a semi-continuous culture investigation revealed that biomass productivity increased up to the 3rd harvest but productivity sharply declined subsequently. The maximum biomass productivity of 0.7 gl⁻¹ d⁻¹ was achieved at the third harvest in the semi-continuous cultivation at initial cell density of 3.92 gl⁻¹.

Key words: arachidonic acid, biomass production, growth optimization, *Parietochloris incisa*, vertical tubular photobioreactors

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Introduction

The freshwater green microalga, *Parietochloris incisa* (Trebuixiophyceae, Chlorophyta), isolated from the snowy slope of Mt. Tateyama, Japan (Watanabe et al. 1996), is capable of accumulating high amounts of arachidonic acid (AA). This microalga is recognized as the richest vegetal sources of AA (Khozin-Goldberg et al. 2002; Bigogno et al. 2002a; b). Under conditions conducive to oil accumulation, such as nitrogen starvation, significant quantities of AA is produced that could exceed 20% of dry weight (Khozin-Goldberg et al. 2002). The proportion of AA reaches 50% of total fatty acids in which over 90% of AA is deposited in triacylglycerols (TAG) (Bigogno et al. 2002a; b). *Mortierella alpina*, the current source of commercial AA, is a zygomycete fungus heterotrophically cultivated in the production of arachidonic-acid-rich oil. It can accumulate up to 40% (w/w) lipid, of which up to 40% can be arachidonic acid when cultivated in submerged culture in a fermenter with glucose as a carbon source (Wynn et al. 1999). Singh and Ward (1997) reported that a fed-batch culture system of *M. alpina* (ATCC 3222) at 25°C can produce a high biomass of 52.4 gl⁻¹ and AA content of 9.1 gl⁻¹ in 8 days.

AA is considered as a nutritionally valuable polyunsaturated fatty acid known to be beneficial supplement particularly for preterm infants. The ability of *P. incisa* to deposit AA in triacylglycerols in cytoplasmic oil bodies is of practical value since TAG is the preferred chemical form for the introduction of AA into the milk formula for the newborn (Cohen 1999).

Biomass productivity, along with biomass content and overall productivity of desired product, is one of the key components affecting commercial feasibility of a microalgal mass cultivation technology. Enhancing biomass productivity at a reduced cost through growth optimization is one of the basic steps in microalgal mass cultivation technology development.

Extensive studies on arachidonic acid synthesis, detailed analysis of lipid and fatty acid composition and growth characterization of *P. incisa* have been reported (Bigogno et al. 2002a; b; Cheng-Wu et al. 2002; Khozin-Goldberg et al. 2002; Solovchenko et al. 2008), however practical growth optimization studies for biomass production are lacking particularly in outdoor conditions. The aim of this work is to increase biomass productivity and improve production process through media optimization, optimal density and harvesting cycle elucidation.
Materials and methods

Organism and culture maintenance

Parietochloris incisa is an isolate from Mt. Tateyama in Japan. Cultures of P. incisa were cultivated under 22±0.2°C in 300 ml flask containing 150 ml Bold Basal Medium (BBM) composed of (per liter): 1.5g NaNO₃, 0.075g KH₂PO₄, 0.175g KH₂PO₄, 0.075g MgSO₄·7H₂O, 0.084g CaCl₂·2H₂O, 0.00498g FeSO₄·7H₂O, 0.05g EDTA, 2 Na salt, 0.025g NaCl, 0.031g KOH, 11.42 µg H₂BO₃, 14.4 µg MnCl₂·4H₂O, 8.82 µg ZnSO₄·7H₂O, 1.57 µg CuSO₄·5H₂O, 0.049 µg Co(NO₃)₂·6H₂O, 0.71 µg MoO₃.

Inocula for outdoor experiments were cultured in 5 liter Erlenmeyer flasks containing 4 liters medium and mixed by air containing 1% CO₂ (v/v) at a flow rate of 0.2 l/min⁻¹. Cultures were maintained by changing medium at two weeks interval.

Vertical Tubular Photobioreactors (VTPR) with 5 cm inner diameter, 2 meters height and a working volume of 4 liters were used for outdoor cultivation. Laboratory grown inoculum was transferred to the VTPR at least 4 days prior to the start of the experiment, unless otherwise stated. Mixing and CO₂ was provided by enriched air with 1% CO₂ (v/v) at the rate of 1 l/min through perforations at the bottom of the reactor. The rate of gas flow was controlled by a flow meter. The temperature was controlled by automatic water mist and temperature did not exceed 30°C.

These experiments were conducted from June to October in Tokyo, Japan. The average daily solar irradiation (kWhm⁻²d⁻¹) per month was 4.21, 4.43, 4.47, 3.32 and 3.05 for June, July, August, September and October, respectively (source: Atmospheric Science Data Center, NASA Surface meteorology and Solar Energy, http://eosweb.larc.nasa.gov)

For media selection, BG11 (Stanier et al. 1971) and BBM were used as basic media and the variation used was twice the concentration of the normal medium 2xBG11 and 2xBBM. Microelements were not doubled in concentrations.

For nitrogen concentration optimization, sodium nitrate was used as nitrogen source at 3, 1.5 and 0.75 g l⁻¹ test concentrations.

Optimal cell densities were evaluated using inoculum grown in the laboratory and inoculum acclimated in outdoor conditions. Cell densities from 1.86 to 4.65 g l⁻¹ at inoculation were used for laboratory grown inoculum and cell densities of 2.34 to 5.4 g l⁻¹ were used for outdoor acclimated inoculum.

In a semi-continuous culture, harvesting was performed on 8, 16, 22 and 29 days of cultivation to determine the number of times cultures can be partially harvested without affecting productivity. Laboratory grown inoculum was used and the cultures were partially harvested to put back the cell density to the initial cell density level.

Growth measurements

Dry biomass

Ten ml of culture aliquot was filtered on tarred pre-dried GF/C Whatman filters. Filtered biomass was washed with 10 ml of acidified distilled water (pH 4) to remove inorganic salts and dried at 105°C for 3 hours. Dried samples are allowed to cool to room temperature in a desiccator over silica gel before being weighed.

Chlorophyll determination

Two ml of culture aliquots were centrifuged at 2000g for 5 minutes. Pigments were extracted from algal pellets with DMSO in 70°C water bath for 20 minutes. The extracts were mixed, centrifuged at 2000g for 5 minutes and measure spectrophotometrically based on Wellburn (1994).

Fatty acid analysis

Fifty ml of culture aliquot were centrifuged at 2000g for 5 minutes and the algal pellets were immediately frozen and freeze dried. Total fatty acid content of the dry biomass was determined following the method of Bligh and Dyer (1959). Freeze dried algal biomass was transmethylated with methanol-acetylchloride and gas chromatographic analysis was conducted following methods of Cohen et al. (1993). Gas chromatography was performed on a fused silica capillary column (100 m x 0.25 mm x 0.2µm). Heptadecanoic acid was added as an internal standard and fatty acid methyl esters were indentified by co-chromatography with authentic standards.

Statistical analysis was done as ANOVA at 95% confidence interval.

Results

BBM and BG11 were used as base inorganic media formulations and nutrient concentrations twice of the normal levels were variants for media selection. The performances of the different media formulations were evaluated at two initial cell densities of 0.8 gl⁻¹ (LD) and 2.3 gl⁻¹ (HD) in the months of September to October. At LD, biomass productivities in different culture media did not vary significantly up to 14 days of cultivation (Fig. 1), however, significant differences in productivity were observed in various media formulations starting from 10 days of cultivation of HD cultures and productivities obtained at the end of cultivation were of 0.25, 0.23, 0.16 and 0.14 g l⁻¹ d⁻¹ for by 2xBBM, BBM, 2xBBG11 and BG11, respectively (Fig. 1).

The optimum nitrogen concentration for biomass production was explored at initial population densities of 0.88 gl⁻¹ using BBM as based medium and nitrogen concentrations were varied between 3 to 0.75 gl⁻¹. This experiment was performed in month of June. Cultures
Changes in chlorophyll content along the cultivation time of *Parietochloris incisa* grown in different sodium nitrate concentrations. Bars represent the standard deviation, n=3.

Discussion

Development of mass cultivation technology for a specific microalga requires media optimization for both biomass production and accumu-
lation of the desired product for maximum productivity. An important consideration in algal mass cultivation aimed at producing high yields of biomass is to ensure that all essential nutrients are supplied in adequate amounts. However, nutrient costs have a great impact on the overall feasibility of an algal mass cultivation system and an estimated range of $\%$ of total production cost in open pond system was reported (Borowitzka 1999). Therefore, the minimal amount of nutrients that must be added to the culture medium which will not be prohibitive to biomass productivity must be identified. This study is basically a step-wise exploration where the best medium formulation was first determined and using the selected medium, the optimal sodium nitrate concentration for biomass production was elucidated. Optimal cell density and harvesting trials were conducted using the selected growth medium at optimal nitrogen concentration.

The inorganic media BG and BBM are commonly used for the cultivation of freshwater microalgae. Performances of BG11, BBM, 2xBG11 and 2xBBM were evaluated for biomass production of P. incisa under LD and HD cultures. The biomass productivities achieved by BG11, 2xBG11 and BBM at LD indicates that these media formulations supplied adequate nutrients to support maximum productivity under this condition. An apparently lower productivity in 2xBBM observed could be attributed to the possibility that the threshold concentration of a certain nutrient could have been exceeded resulting in reduced productivity. A different growth profile was observed at HD cultures where productivities significantly differed in all media formulations. The lower productivity in BG11, 2xBG11 suggests nutrient deficiency as supported by the observation that during the course of cultivation, BG11 consistently supported a lower biomass while 2xBBM decreased biomass productivity only from day 10 indicating that nutrient deficiency could have occurred at this point limiting the al-

Fig. 4  Biomass productivity of *Parietochloris incisa* with different initial cell densities using (a) laboratory grown inoculum and (b) outdoor grown inoculum. Bars represent the standard deviation, n=3.

Fig. 5. Change in biomass productivity of *Parietochloris incisa* under semicontinuous cultivation. Bars represent the standard deviation, n=3.
gal growth. The high N: P ratio of BG11 (45:1) could also have rendered the HD cultures P-limited. The specific limiting nutrient could not be elucidated under these experimental conditions. Result indicated that 2xBBM was optimum medium for HD cultures; however, upon considering the high cost of nutrients, the small increase in biomass yield in 2xBBM seemed not justified and therefore BBM was selected as the base medium for *P. incisa* biomass production.

Nitrogen is one of the most important nutrients contributing to the biomass production of microalgae and could greatly affect the productivity of the culture. In a production system aimed for high biomass yield, it is essential to supply sufficient amounts of nitrogen since even under nitrogen-sufficient conditions, microalgae have a limited capacity to store nitrogen in storage bodies, except for cyanobacteria (Boussiba and Richmond 1980; Simon 1971).

Sodium nitrate concentration in the medium at 3 and 0.75 gl⁻¹ resulted in a lower cell density and productivity. The biomass productivity of *P. incisa* was enhanced significantly when sodium nitrate concentration was increased from 0.75 to 1.5 gl⁻¹, however, at concentration of 3gl⁻¹, a critical threshold concentration for this nutrient might have been reached resulting in a reduction in growth. This seemed contradictory with chlorophyll content obtained at 3 gl⁻¹, where higher chlorophyll content was reached compared to 1.5 gl⁻¹ but did not equate to higher growth. Doubling of cellular chlorophyll does not bring about a doubling in the light absorption (Dubinsky et al. 1995) due to the decrease in chlorophyll cross section which actually reduces the light absorbed (Vonsksh and Torzillo 2004). High chlorophyll content is an adaptation to low light, and the high chlorophyll content could also have led to the occurrence of photoinhibition in the cells. At 0.75 gl⁻¹ NaNO₃, N-limited condition could have occurred and one of the typical responses of microalgae to N-deficiency is the reduction in chlorophyll production due to inhibitory effect on protein biosynthesis. Furthermore, the observation that biomass in 0.75 and 1.5 gl⁻¹ sodium nitrate increased at the same rate up to the 6th day of cultivation and growth gradually diverged resulting in culture with 1.5 gl⁻¹ sodium nitrate attaining a significantly higher productivity at the end of the cultivation period supports the occurrence of nitrogen limitation in 0.75 gl⁻¹. The sodium nitrate concentration of 1.5 gl⁻¹ was optimal for biomass production under the existing experimental conditions outdoors.

The basic principle of outdoor microalgal mass cultivation technology is to efficiently utilize the strong solar irradiation for photosynthetic biomass productivity along with secondary metabolites and theoretically, growth of microalgae must be limited by light only (Richmond 2004). Factors that affect the culture productivity as related to the amount of light received by individual cells in the culture are length of light path of the photobioreactor, culture turbulence and cell density. In this study, the light path was fixed and the effective turbulence was pre-determined to prevent cell sedimentation as well shearing of cells. The optimal cell density was thus elucidated.

The biomass productivities at different cell densities were evaluated. Results clearly demonstrated that at a given light intensity, i.e., full sunlight, the biomass productivity of *P. incisa* was enhanced as the population density increased from 1.8 to 4.65 gl⁻¹ in the laboratory grown inoculum and similarly, in outdoor-acclimated inoculum, from 2.34 to 4.31 gl⁻¹. A reduction in productivity was observed at 5.4 gl⁻¹ in the outdoor acclimated inoculum. The lower productivity at high cell concentration was attributed to severe shortage of light available to individual cells since the population density determines the depth at which the light can penetrate into the culture. As the cell density increased, the more energy is trapped at the surface of the reactor blocking the light penetration. On the contrary, cell densities below optimal cells are exposed to excessive light that result in light induced depression of photosynthesis or photoinhibition. Excess light can be potentially harmful and low densities could lead to the total collapse of the culture within a few hours after transfer outdoors (Hu and Richmond 1994). The mutual shading of cells at optimal cell density protects the culture from photoinhibition and facilitates the most efficient exploitation of light reaching the culture. Richmond (2004) explained that if a well defined optimal density is not achieved, the culture is not light limited and other growth factors that are not optimal may be limiting the culture productivity. In this study, the optimal density was derived at cell density range of 4.31–4.65 gl⁻¹ and this indicates that the other growth factor particularly nutrient availability was optimal.

The biomass productivity of *P. incisa* inoculated from the laboratory to outdoor was evidently lower as compared to the productivity of outdoor photoacclimatized cultures. The drastic change in light intensity when the culture was transferred from the laboratory to outdoor resulted in the overall reduction in biomass productivity caused by photoinhibition. Though, microalgae have a great capacity for photoacclimation, however, these process takes time and involves physiological biochemical and ultrastructural changes (Falkowski 1980; Escoubas et al. 1995; Fisher et al. 1996). This initial low biomass productivity of low light adapted laboratory grown *P. incisa* mandates that a steady supply of outdoor acclimated cultures be made available.

It was observed that regardless of nutritional levels, the culture productivity of *P. incisa* generally declines after 9 to 10 days of inoculation. Therefore, it is necessary to devise an optimal harvesting cycle to maintain productivity of culture in outdoor biomass production. Harvesting was performed between 6-8 days of cultivation, before stationary phase of growth was reached. The biomass productivity gradually increase as the cells become acclimatized to high light intensity in outdoor conditions and attained maximum productivity on the third harvest. However, the effect of cell density on the biomass productivity
was not clear on the first two harvests mainly due to the intermittent low light intensity during the first two weeks of cultivation as affected by weather conditions. The weather improved in the third and fourth week of the experimental period. However, after only three harvests, the productivity dropped at the fourth harvest, regardless of cell density. Factor such as nutrient levels, and light limitation was considered. However, since the same observation was obtained at all cell densities, nutrient and light limitations were ruled out. The reduction in productivity may possibly be attributed to inhibitory substances excreted into the medium which could have accumulated through the culture period. These algal metabolites were reported to inhibit their own species growth, as well as other species. Growth of *Chlorella vulgaris* was reported to be depressed by its own product, chlorellin, excreted into the medium (Prat and Fong 1940). It was also reported that dense culture of *Skeletonema costatum* may inhibit its own growth (Curl and McLeod 1961). Moreover, *P. incisa* was reported to produce a substance excreted into the medium that acts like an auto-inhibitor and is reported to decrease cell growth, chlorophyll, carotenoids, proteins, TFA and AA production (Liu et al. 2002). This substance was usually reported in ultra high density cultures reaching up to 50 g l⁻¹, however in *P. incisa*, it seemed that regardless of density, the inhibitory effect was evident. Cultures in all different cell densities exhibited the same rate of productivity decline. This may impose a problem in mass cultivation if the cultures are not properly managed. Hu et al. (1998) reported that in *S. platensis*, high cell densities up to 50 g dry weight per liter culture was achieved in a 1-2 cm optical path photobioreactor at 4000 µEm⁻²s⁻¹ only if the entire growth medium was replaced daily and simply adding full nutrient medium did not have significant effect on growth.

It could thus be concluded that medium optimization is required in the development of mass cultivation technology of a specific microalgal species and that simply supplying nutrient in excess will not necessarily result in high biomass productivity. Optimal nutrient concentrations will not only increase productivity but will also cut down production cost. Based on the combined result of these experiments, BBM with 1.5 g l⁻¹ sodium nitrate at initial cell density range from 3.92 to 4.31 g l⁻¹ were optimal for biomass production under the experimental condition described in these study where productivities of 0.6-0.7 g l⁻¹ d⁻¹ was attained. Outdoor inocula cultures can sustain productivities up to three harvests before the entire medium must be replaced. This is of particular significance in the operation of large volume of cultures in a mass production facility. Frequent changing medium may pose a risk of exposing cultures to contaminations as well as additional operation cost. Optimization of growth considering the environmental conditions as affected by climate must be undertaken year round in order that the full potential of the algal species will be fully realized.

References


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