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Growth Characteristics of Ultrahigh-density Microalgal Cultures

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Abstract The physiological characteristics of cultures of very high cell mass (*e.g.* 10 g cell mass/L), termed "ultrahigh cell density cultures" is reviewed. A close relationship was found between the length of the optical path (OP) in flat-plate reactors and the optimal cell density of the culture as well as its areal (g m^{-2} day^{-1}) productivity. Cell-growth inhibition (GI) unfolds as culture density surpasses a certain threshold. If it is constantly relieved, a 1.0 cm OP reactor could produce *ca.* 50% more than reactors with longer OP, *e.g.* 5 or 10 cm. This unique effect, discovered by Hu et al. [3], is explained in terms of the relationships between the frequency of the light-dark cycle (L-D cycle), cells undergo in their travel between the light and dark volumes in the reactor, and the turnover time of the photosynthetic center (PC). In long OP reactors (5 cm and above) the L-D cycle time may be orders of magnitude longer than the PC turnover time, resulting in a light regime in which the cells are exposed along the L-D cycle, to long, wasteful dark periods. In contrast, in reactors with an OP of *ca.* 1.0 cm, the L-D cycle frequency approaches the PC turnover time resulting in a significant reduction of the wasteful dark exposure time, thereby inducing a surge in photosynthetic efficiency. Presently, the major difficulty in mass cultivation of ultrahigh-density culture (UHDC) concerns cell growth inhibition in the culture, the exact nature of which is awaiting detailed investigation.

Keywords: ultrahigh density culture, flat plate reactor, optical-path, growth inhibition, L-D cycle, productivity

INTRODUCTION

This work addresses the growth response of phototrophic microalgae cultivated under unique conditions *i.e.* flat plate reactors with a very short optical paths (OP) of 1 to 2 cm, in which very high cell densities (10 to 20 g/L dry cell mass) were exposed to strong (1,000 to 4,000 μmole photons m^{-2} s^{-1}) light.

Javanmardian and Palsson [1] pioneered the method for culturing very high cell densities exposed to strong light. Aiming to reach maximal photosynthetic productivity, they designed a photobioreactor system in which an optical transmission system based on fiber optics illuminated the culture from inside. Significantly, an online ultrafiltration unit which exchanged spent- with fresh medium was mandatory for obtaining the very high cell density reached – *ca.* 30 g /L or 10^9 cells/mL of *Chlorella vulgaris*. The reported output rate was 63 mg of dry *Chlorella* L^{-1} h^{-1}. As shall be elucidated further on in this work, applying external illumination (900 μmole photons) to a flat plate reactor of 7.5 mm (OP) of *Spirulina* sp. culture, Hu *et al.* [2] obtained 621 mg L^{-1} $h^{-1}$and, in a 14 mm OP flat plate illuminated externally at the rate of 8,000 μmole photons, they harvested 1,200 mg L^{-1} h^{-1}.

The practical advantages of using cultures of very high cell density may be readily seen: In addition to the obvious benefit of possibly obtaining significantly higher output rates of cell-mass per reactor volume and illuminated area, there is the overall saving of investment costs involved in a smaller reactor system. Operating costs should also be reduced, smaller culture volumes reducing the costs of culture maintenance as well as harvesting the cell mass, a concentrated cell mass being, as a rule, easier to recover in high efficiency. The reduction in the investment costs per unit product is further augmented by the saving in infrastructure as culture volumes required to produce a given amount of product become smaller.

INTERRELATIONSHIPS BETWEEN THE OPTICAL PATH (OP), CELL DENSITY AND PRODUCTIVITY

The study of the growth physiology of ultrahigh cell density cultures (UHDC, a term coined by Hu *et al.* [3] for cultures with cell concentration of at least 10 g dry-cell mass per L or 150 mg chlorophyll/L), received strong impetus with the discovery of Hu *et al.* [3] that a radical reduction in the optical path (OP) of a flat plate

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reactor affected a significant increase in the areal productivity (g m^{-2} day^{-1}) of continuous *Spirulina* cultures. The optimal cell density (OCD, *i.e.* yielding the highest net areal productivity) of a 10.4 cm (OP) reactor placed outdoors was 1.7 g/L, the areal yield, calculated on the basis of the reactor's front panel reaching 33.6 g m^{-2} day^{-1}. An eightfold reduction of the OP to 1.3 cm resulted in an increase of *ca.* 50% in the output rate, yielding under exactly the same conditions, 51.1 g m^{-2} day^{-1}.

Similar laboratory experiments, in which the range of optical paths span from 20.0 to 0.75 cm, (for *Spirulina* sp.) or 9.0 to 1.0 cm (*Nannochloropsis* sp.), gave very much the same results, the areal output rate of the 0.75 cm or 1.0 OP reactor being ca. 50% higher than that obtained in the 20 or 9 cm reactors [2,4].

A basic feature of UHDC must now be put in focus: The response of increased areal productivity to an extreme reduction of the OP was observed only when growth inhibition (GI - to be alluded to in a later section), was prevented from building up in the culture , by replacing daily the entire culture medium with fresh growth medium. The work of Zhang, detailed in Richmond *et al.* [4], elucidates the interrelationships between the OP and GI. In a 1.0 cm OP flat plate reactor with *Nannochloropsis* sp., from which GI was alleviated by a daily replacement of the entire culture medium, the optimal cell density was 7.5×10^9 cells/mL and the output rate was 260 mg L^{-1} h^{-1}. Under identical growth conditions and illumination, the optimal cell density was only 1.0×10^9 cells/mL in the 9.0 cm OP reactor, yielding an output rate of 180 mg L^{-1} h^{-1} (these figures have been corrected for the independent effect reducing the optical path exerts on reduction of the areal volume (cell mass /m^2) and thereby on a proportional increase in volume-yield, g L^{-1} h^{-1}). In case GI in the culture was not addressed *i.e.* culture media was not replaced and only nutrients were supplemented, the interrelationships between the OP and the output rate were reversed: The 9.0 OP reactor yielded twice as much cell mass per illuminated area compared with the 1.0 cm OP reactor, in which the concentration of GI or the extent of growth inhibition were highest [4].

Clearly, when there is no GI built-up in an UHDC, extreme reduction of the optical path to *ca.* 1.0 cm results in an increase of the optimal population density, proportional to the reduction in the OP. A significant surge in the areal productivity of cell mass follows, reflecting the substantial rise in photosynthetic efficiency. In what follows, this phenomenon is elucidated.

INTERRELATIONSHIPS BETWEEN THE FREQUENCY OF THE LIGHT- DARK CYCLE (L-D) IN THE REACTOR AND THE PHOTOSYNTHETIC CENTER (PSC) TURN-OVER TIME

In UHDC, the extent of light absorption by the culture as well as the extent of mutual shading is such that irradiance impinging on the reactor surface attenuates rapidly, resulting in formation of a relatively small photic volume in the reactor. The depth light penetrates into the culture is a function of cell density, Janssen *et al.* [5] having computed the lit fraction of reactor volume in a 1.3 cm OP reactor with a cell concentration of 10.0 g/L to be only 0.062. The frequency of the light-dark (L-D) cycle cells in UHDC undergo in their continuous travel across the optical-path assumes therefore great significance. In an attempt to explain the surge in photosynthetic efficiency taking place as the OP is reduced to *ca.*1.0 cm or less, it is useful to focus on the timescales discernible in the photosynthetic reaction, *i.e.*, the light reaction time, of the order of ns, and the dark reaction time, of the order of 1~15 ms. The former is so short that the light reaction may be viewed as instantaneous. Hence, the photosynthetic unit (PSU), or photosynthetic reaction center turnover time is, for all practical purposes, equal to the dark reaction time [6,7].

Cell travel time represents the average time required for cells to move back and forth between the photic and dark reactor volumes, cell motion in a reactor stirred by air being the consequence of the turbulent fluid motion induced by the passage of air bubbles. Expecting the actual cell motion to be some combination of regular- and random motion, rough estimates indicate the real L-D cycle time for an optical path of 6 cm lies between 200 and 6,000 ms, for orderly and random cell motion, respectively [8]. For an OP of 1 cm however, L-D cycle times range between 33 and 167 ms for orderly and random motion, respectively. As the optical path is further reduced to an extreme of 0.375 cm, L-D cycle times of orderly and random cell motion would be 12 and 23 ms, respectively, assuming a photic volume of 5% and a fluid velocity of 30 cm/s. Hence indication that cell travel time begins to represent a relevant parameter for enhancement in photosynthetic productivity not before the optical path is reduced to *ca.* 1.0 cm or less.

In the work of Hu *et al.* [9], cell travel times in the 10.3 cm OP reactor may be orders of magnitude greater than the timescale of the complete photosynthetic reaction or the PSU turnover. In this case, timescales should be altogether ignored, culture productivity being dependent on the intensity of the light source as well as on the exponential decline of radiation intensity (modulated by cell density) across the optical path. Under these circumstances, which in fact predominate in micro-algaculture, the volumetric productivity (g L^{-1} h^{-1}) is inversely proportional to the light path, and the areal density as well as photosynthetic productivity, do not change as the length of the optical path is manipulated. This has indeed been observed in experiments in which the optical path was much reduced but not to lower than 10 cm [10].

In the 1.3 OP reactor, in which a 45% surge in areal yield was observed compared with the 10.3 OP reactor, cell travel times (L-D cycle) begin to approach the turnover time of the photosynthetic unit [6]. Since only a small fraction of the cells (5~10%) at any given instant is exposed to irradiance sufficient for photosynthesis, the cells in the reactor are necessarily exposed to wasteful dark periods. The length of the dark period in an L-D cycle may thus be practically targeted for improving the

light regime in order to increase photosynthetic productivity. It is because the timescale of the PSU-photosynthetic reaction center-turnover rate (equal to the dark reaction time) and the timescale of cell travel across the rector walls (the L-D cycle), assume values which approach the same order of magnitude in high cell-density short optical path reactors, that the photosynthetic efficiency of the culture is augmented as observed by Hu *et al*. [2,3,9] and Richmond *et al*. [4].

It follows that the shorter the optical path (up to a point), the closer would be the synchronization between the residence time of cells in the dark zone and the PSU turnover time, and the higher should become light-use efficiency and photosynthetic productivity. This assertion, however, must yet be empirically tested in detail.

Summing up; the OP must be reduced to *ca*. 1 cm for a marked reduction in the wasteful dark fraction of the L-D cycle to take place. In contrast, reduction of OP to 10 or even to 5 cm is not accompanied with a meaningful decrease in the wasteful dark residence, and therefore has no effect on areal productivity.

EFFICIENCY OF LIGHT-USE IN UHDC

Light impinging on culture surface is best utilized, *i.e*. yielding highest photosynthetic efficiency, when culture cell density is optimal [11]. In general, when cell density is significantly bellow optimal, light is not only wasted, but if applied in great excess, may affect photoinhibition [12], culminating, in extreme cases, with cell death. In cultures with cell densities significantly above optimal, cells are severely light-limited, resulting in slow growth due to a shortage in carbon skeletons coupled with high maintenance energy expenditure as well as high respiratory losses.

Photoinhibition does not take place in UHDC or may only occur to a slight extent, even under very strong light, *e.g*. 4,000 μmole photons $m^{-2} s^{-1}$ [2], providing the OCD is maintained and the cells have been acclimated to high light. This may be readily explained in that the extreme self-shading in UHDC greatly diminishes the light dose available to the individual cells. Indeed, the photosynthetic efficiency, which generally declines rapidly when light intensity increases, did not fall in UHDC of *Spirulina* sp. up to a photon flux density (PFD) of 2,000 μmole photons $m^{-2} s^{-1}$. This was possible because cell density was adjusted to each increase in light intensity, *i.e*. culture density was maintained at its optimal all along the rise in light intensity [2]. Recently, Torzillo *et al*. [13] observed that outdoor *Spirulina* cultures acclimated to strong light and kept at optimal temperature as well as low oxygen, were not photoinhibited even in midday, as evidenced in the constant F_v/F_m ratio observed throughout the day.

The relatively high photosynthetic efficiency displayed in UHDC may be explained in that the high photon flux reaching the culture surface is in effect much diluted, being shared by a great number of cells, each receiving only a small light dose. The greatly diminished light dose available for each cell in UHDC, is compensated in that it is utilized at the highest efficiency, falling on the linear phase of the PI curve. This effect, together with the greatly increased frequency of the L-D cycle due to the shortened OP, result in the observed efficient use of strong light, evidenced in a higher light-yield [4].

MIXING OF UHDC: IMPLICATIONS ON PRODUCTIVITY AND HYDRODYNAMIC STRESS

The importance of providing mixing in relatively dense microalgal cultures is well recognized, but in UHDC, the mode and the rate of mixing are of particular significance.

The boundary layer gradients associated with the gaseous and molecular exchange taking place between the cell and its environment in the course of photosynthesis and growth, become ever more growth-limiting as cell density increases. The volumetric mass transfer coefficient (k_La) in the culture should therefore be sufficiently high to bar oxygen build-up to growth- inhibitory pressures as well as insure carbon dioxide sufficiency. Conceivably however, the volumetric mass transfer coefficient may become very low in UHDC, inhibiting growth. In fact, k_La was reported to decrease in *Spirulina* culture grown in flat plates from *ca*. 50 at cell density of 5 g/L to *ca*. 20 at cell density of 35 g/L [3]. As has been already documented by Markel [14] and by Richmond and Grobbelaar [15], cultures of relatively low cell densities require, as a rule, low mixing rates and culture productivity is essentially not dependent on the rate of mixing. In contrast, cell-mass production rate of UHDC of *Spirulina* sp. exposed to strong light in flat plates was very sensitive to the velocity of air used for mixing; the output rate of *Spirulin*a cell mass doubling (to 400 mg $L^{-1} h^{-1}$) as air flow (L air L^{-1} culture min^{-1}) was increased from 0.6 to 4.2. The fact that when the flow of air in that culture was further increased to 6.3, maximal productivity was reduced by some 30% indicating cell damage, requires attention:

According to Barbosa [16], cell damage may take place during bubble formation, bubble rising or bubble breakup. Very few quantitative studies are available concerning hydrodynamic stress in microalgal cultures cultivated in gas-sparged photobioreactors, but recently, Barbosa *et al*. [17] concluded that bubble formation is responsible for cell damage, cell-death increasing with increasing gas entrance velocity beyond a certain critical, strain-depended value. Indeed, microalgae species vary greatly in sensitivity to cell damage induced by shearing forces, caused by pumping and mixing devices along the production process. All of which, therefore, should be carefully optimized in UHDC with respect to the mode and rate of cell transport and mixing.

Finally, it is worth noting that UHDC have been grown and researched mainly in flat plate photobioreactors. Clearly, mass transfer is substantially greater in pneumatically agitated vertical reactors, such as bubble columns and flat panels, in which the oxygen path is very short, than in horizontal tubular reactors [3,18] Indeed,

the overall volumetric gas-liquid mass transfer coefficient in bubble columns has been estimated to be 4-fold the estimated value for a horizontal tube [18,19], rendering tubular reactors unsuitable for the cultivation of UHDC [16].

GROWTH INHIBITION IN UHDC

An essential characteristic of UHDC which awaits detailed research concerns the inhibition of growth which unfolds as cell density increases over a species- specific threshold.

Attempting to obtain highest photosynthetic productivity as well as maximal light-use efficiency, Javanmardian and Palsson [1] discovered that the very high photosynthetic productivity and efficiency they were seeking could be attained only when an on-line ultra-filtration exchanged spent- with fresh growth medium. Likewise, Richmond *et al*. [4] showed that removal of GI was mandatory for achievement of high light yield and productivity of cell mass. The question naturally rises - what must be replenished or filtered out of the growth medium for achieving and maintaining UHDC?

Two general possibilities are suggested; one concerns growth inhibitory substances exuded from the cells or formed in the growth medium surrounding the cells. The other possibility is that cell growth is curtailed due to nutritional imbalances unfolding when cell density greatly increases. Although there is convincing evidence for the latter possibility, most of the experimental evidence supports the first possibility accordingly, growth inhibitory substances are produced and exuded by algal cells grown to very high densities, *e.g*. Pratt [20], Leving [21], Von-Denffer [22], Lefevre [23], Fogg [24], Harris [25], Keeting [26], all reporting algae inhibitors in culture filtrates of several algal species. In addition, Pratt and Fong [27] observed that growth of *Chlorella vulgaris* was depressed by its own product (*chlorellin*) excreted into the culture medium. Likewise, Curl and McLeod [28] reported that dense cultures of *Skeletonema castatum* may inhibit their own growth. McCracken *et al*. [29] assayed anti-algal substances in the culture medium of *Chlamydomonas reihardtii*, and identified the active substances as linoleic and linolenic acids. Imada *et al.* [30, 31] identified an inhibitor, 15(*S*)-hydroxyeicosa pentaenoic acid, probably an oxygenated metabolite of eicosapentaenoic acid, in cultures of *Skeletonema costatum*. Quantitative aspects of the inhibitory activity in *Nannochloropsis* sp. were determined by Zhang and Richmond (unpublished) who developed a bio-assay for testing the supernatant of 20-day-old stationary phase UHDC of *Nannochloropsis* sp. [4]. Bio-assayed filtrates from this culture exhibited considerable inhibitory activity, which increased sharply as cell concentration rose to 3×10^9 cells/mL, reaching maximal growth inhibition as cell concentration began to decline in the late stationary phase.

Mandalam and Palsson [32] dismissed the postulated existence of specific auto-inhibitors and in a later study [33], suggested that inhibition of cell division in UHDC was due to an imbalance of nutrients: N-8 medium, commonly used for culturing *Chlorella vulgaris* in their experiments was evaluated for its capacity to support high-density cultures based on the elemental stoichiometric composition of *C. vulgaris.* Their analysis indicated N-8 medium became deficient in iron, magnesium, sulfur, and nitrogen, arresting growth as cell density reaches a certain high level. The medium was redesigned (M-9) to contain stoichiometrically balanced quantities of the four deficient elements to support a high cell mass concentration of 2% (v/v). Replacing N-8 medium with the M-9 medium resulted in up to three- to five fold increase in total chlorophyll content per culture volume. Addition of each of the four elements separately to the N-8 medium did not improve culture performance; the balanced supplementation of all four deficient elements being mandatory to yield the greatly improved performance. Hence, support for the possibility the performance of UHDC can be significantly enhanced by re-designing the growth-medium to sustain very high cell densities. Hu *et al*. [2] however, reported *Spirulina platensis* cultures grown in 1.4 cm OP and exposed to 4,000 μE $m^{-2} s^{-1}$ would reach well over 50 g dry weight per liter culture only if the entire culture medium was replaced daily; simply adding the full nutrient medium formula instead of entirely replacing it - did not alleviate growth inhibition. Finally, there is some evidence which indicates both nutritional imbalance and inhibitors may exert combined effects on growth inhibition in UHDC [4].

In summary, there is considerable evidence that sustaining UHDC and reaping the high photosynthetic productivity obtainable in such cultures requires relief from growth inhibitory substances and/or provides carefully balanced nutrient media necessary for sustaining such cultures. In order to become economically feasible, UHDC would require practical means with which to prevent the build-up of inhibitory activity or conditions which arrest cell division and growth.

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