OPTIMIZATION OF *PORPHYRIDIUM PURPUREUM* CULTURE GROWTH USING TWO VARIABLES EXPERIMENTAL DESIGN: LIGHT AND SODIUM BICARBONATE

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Această lucrare are ca principal scop optimizarea condiţiilor specifice de cultură ale tulpinii Porphyridium purpureumin în vederea îmbogăţirii masei algale în componenţi utili, precum phycobiliproteinele şi exo-polizaharidele, creşterea randamentului în masa verde şi a calităţii produselor obţinute prin prelucrarea acesteia.

Datele experimentale de creştere şi formare a bioproduselor în masa verde a microalgei unicelulare Porphyridium purpureum au fost colectate în urma unui experiment cu două variabile independente: iradianta şi concentraţia $NaHCO_3$, alimentat suplimentar în mediul de cultură ASW.

Mărirea iradiantei şi a concentraţiei $NaHCO_3$ în mediul de cultură ASW au condus la creşteri substanţiale în producţia de biomasă, precum şi în randamentele de formare a exo-polizaharidelor. Iradianta este factorul determinant în acumularea ficobiliproteinelor în masa algală. Concentraţiile maxime ale ficobiliproteinelor identificate în masa algală uscată sunt: Focoeritrina 12.17%, R-Ficocyanina 10,2 %, Aloficocyanina 2.86%.

This paper concerns the optimizing Porphyridium purpureum culture growth under specific helpful conditions in order to promote the value added products, as phycobiliproteins or exo-polysaccharides, increasing yields and products quality.

Experimental data of growth and bio-product formation by the unicellular microalga Porphyridium purpureum, using two variables experimental design: light and sodium bicarbonate feeding through amending ASW nutrient medium with additional amounts of $NaHCO_3$, have been presented.

More irradiance intensity and $NaHCO_3$ in ASW medium has led to substantial increases in the biomass production, as well as in the exo-polysaccharide yields. Irradiance is a determining factor in the high phycobiliprotein accumulation.

The maximum concentrations of individual phycobiliprotein content in dry mass are as follows: Phycoerythrin 12.17, R-Phycocyanin 10.2, and Allophycocyanin 2.86 (% on the dry biomass basis).

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Keywords: Porphyridium purpureum, Microalgae, Biomass, Phycobiliproteins, and Polysaccharides

1. Introduction

Porphyridium purpureum is a unicellular red microalga from Rhodophyta class which has the potential to crop large amounts of proteins (28-39%), polysaccharides (40-57%) and lipids (9-14%) subsumed into dry algal mass [1]. This strain is unique in building up fluorescent phycobiliproteins, exopolysaccharides, long-chain polyunsaturated fatty acids, carotenoids (zeaxanthin, tocopherol, etc.) and vitamins during its metabolic processes [2-4]. It has been shown that *Porphyridium purpureum* contains four phycobiliproteins with the following concentrations in the dry mass: allophycocyanin (5%), R-phycoerythrin (11%), b-phycoerythrin (42%), and B-phycoerythrin (42%). Beside the polysaccharides sustaining antiviral activity, B-phycoerythrin has appreciable value in certain applications, due to its particular high molar absorptivity and enlarged fluorescence level [2, 5, 6]. Culture conditions such as light intensity and residence time were reported to influence the phycobiliprotein concentrations and ratios [7, 8]. Porphyridium purpureum 337 cells produce three types of sulphated polysaccharides, namely intracellular polysaccharides, pericellular polysaccharides and hydrosolubilized polysaccharides with a molecular weight of 3-5 x 10^6 Da. This indicates that the raw material might be processed to excellent green biolubricants [7]. The hydrosolubilized polysaccharides are dissolved in culture medium and are called exo-polysaccharides [9]. Also, the algal enriched material is rich in PUFA and contains about 33% arachidonic acid. Up to 17% of total fatty acids are EPA, which may be used as supplements for health care [10, 11]. For optimal EPA production in *Porphyridium purpureum* growth, the temperature of *culture medium* has to be hold below 25°C [12]. Meaningful data are reported about the growth of *Porphyridium purpureum* in artificial seawater medium (ASW medium) [13, 14].

2. Experimental

Inoculums and culture media. Inoculums of *Porphyridium purpureum* was originally obtained from the collection of algal strains of University Babes Bolyai, Cluj Napoca, Romania [15]. Ten days old algal culture, with optical density (OD) values of 0.05 – 0.10 units, was used as inoculum in all the experiments (time t_0 of the experimental exponential growth). Cells were grown in the 3.0L batch culture of photo-bioreactor (type PBR2S, Sartorius, Germany), using artificial seawater as medium with the following composition:15.0g/L NaCl; 3.05g/L $MgSO_4 \cdot 6H_2O$; 2.8g/L $MgCl_2 \cdot 6H_2O$; 0.75g/L $CaCl_2 \cdot 2H_2O$; 1.0g/L KNO_3; 0.08g/L KH_2PO_4;

0.54g/L $NaHCO_3$ and 1 mL/L of trace metal solution (2.8 g/L H_3BO_3, 2,03 g/L $MnSO_4 \cdot 4H_2O$, 0.222 g/L $ZnSO_4 \cdot 7H_2O$, 0.018 g/L MoO_3 (85%), 0.079 g/L $CuSO_4 \cdot 5H_2O$ and 0.494 g/L $Co(N)_3)_2 \cdot 6H_2O$), previously mixed with 1 mL/L of chelated iron solution (0.69 g $FeSO_4 \cdot 7H_2O$ + 0.93 g Na_2EDTA in 80 mL demineralized water) and adjusted to pH 7.4. The above ASW standard medium was amended by adding sodium bicarbonate up to 3 g/L, as one of the variables for optimizing the culturing conditions in order to control both the accumulation of desired products in the algal biomass and the exo-polysaccharides in nutrient culture medium.

Growth data collection. The photo-bioreactor was operated as a batch reactor, feeding the previously aged inoculums at moment t_0 of the experiment, when all measurements were started. Because the seedling charges of inoculums are just a fraction from the total volume of fluid in reactor, the first optical density measurement OD_0 is taken immediately after the inoculums seeding. Further, all the measurements were done daily and one day is set as the time unit. All the parameters, i.e. OD (optical density), pH, temperature, time, light intensity and CO_2 flow rates were continuously plotted by photo-bioreactor sensors and soft.
Experimental data analysis was based on Guillard [16] theory of the exponential phytoplankton population growth, adapted by Wood et al [17] for algal growth in common photo-bioreactors. According to Wood et al. [17], the rate of cell number growth at moment t_i is proportional with the number of cells N_{ti} at that moment in the available culture medium, i.e. workable reactor volume. Therefore, the process kinetics is described by equation:

$$dN/dt = R_{exp}\ N \tag{1}$$

Because the seedling inoculums already reached the exponential growth at the moment t_0, R_{exp} is the variation of cell number in one unit of time at an arbitrary moment t divided by the total cell number in the reactor volume at the end of elapsed time unit, or the exponential rate of cell population growth expressed in unit t^{-1} (actually day^{-1}). Integration of the equation (1) from t_0 to t (end moment of the exponential growth) evaluated from the experimental data results in:

$$N= N_0 \exp (R_{exp}t) \tag{2}$$

where N is the total number of cells in the workable reactor volume at the end of exponential growth. Replacing N and N_0 by the measurable equivalent parameters OD and OD_0, a new equation may be written and experimental data may be used to compute the exponential growth rate of algae under particularly specified conditions:

$$\ln OD/OD_0 = R_{exp}t \tag{3}$$

Beside the exponential growth rate (in OD units/day), some other data useful for evaluation of the variable process parameter effects on rate and yield of the algae culture might be derived from equation (3), as follows:

$$T_D = 0.6931 / R_{exp} \quad (4)$$

where T_D is the doubling time of cells number expressed in days, and

$$N_D = R_{exp} / 0.6931 \quad (5)$$

where N_D is the doubling number of cells per day.

The experimental growth curves were plotted on semi-logarithmic graphs ln OD / OD_0 against exponential growth time like in Fig. 1. Interpretation of these graphs should be regarded as a quite disputable subject. The OD_0 point can move to great extent along the Y axis, mainly due to the uncertain exponential rate approach of the growth process at t_0 moment. In other words, there is a time lag between seeding the inoculums into culture media and the beginning of true exponential growth rate in the growth volume. This happened obviously, despite the OD_0 record is made simultaneously with the inoculums seedling admixture.

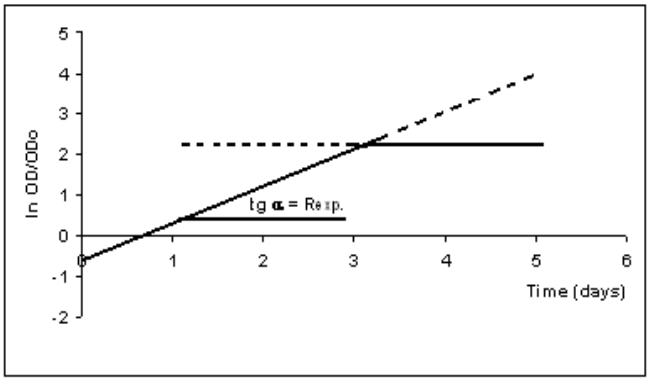

Fig. 1. Hypothetical graph ln OD/ODo versus time

In one full set of experiments the OD_0 position may vary randomly with regard to the expected effects induced by one or more growth process parameters. In this case, the better course is to disregard the OD_0 point read at the moment t_0 or disregard the experimental data collected after the first day. For the other points fitting on the graphs ln OD/OD_0 against exponential growth time, the best approach for an accurate exponential rate of growth computation is the data regression taking ln OD/OD_0 =1.0 as the first point at t=0. Other authors recommend the choice of the best accurate two points on the graphs, and further proceed with the exponential rate of growth calculation from the equation:

$$R_{exp} = \ln (OD_2 / OD_1)/(t_2 - t_1) \quad (6)$$

Irradiation parameters. Cultures were continuously irradiated with fluorescent lamps under un incident intensity of 120 and 240 $\mu E/m^2 s$ at a constant temperature 22^0C. Culture media were mixed by recycling the fluid mass with a peristaltic pump and by bubbling air with 7.0% (v/v) CO_2 content.

Algal mass harvesting. Algal cultures were harvested after 12-16 days of growth. Algal mass was separated by centrifugation at 4000 rpm and the production was reported in terms of dry weight (g/L). Harvested cells were washed twice with distilled water and then were processed by lyophilization at -50^0C and 0.011 mbar (ALPHA 1-2 LD_{plus} CHRIST freeze-dried).

The main constituents of algal biomass were evaluated by comparison with standard constituents, using thermal analysis (Termogravimetric Analyser 951, Du Pont Instruments 2100) and FTIR analysis (FTIR 6300 – Jasco provided with ATR Golden Gate – Diamond/Sapphire).

Phycobiliproteins evaluation. Dry algal biomass (1 g) is extracted, 5 times, each with 20 mL 0.1 M phosphate buffer (pH 6.8) in an ultrasonic bath for 5 minutes. Each time the supernatant was separated by centrifugation. Phycobiliprotein concentration was determined in supernatant by spectrophotometry anaysis. The absorbance of extracts was measured at wavelengths 565, 620, and 650 nm using Spectrophotometer UV-VIS Specord M400 Karl Zeiss Jena, and concentrations were calculated using equations 7-9, [18]:

$$\text{Phycoerythrin (mg/mL)} = [\varepsilon_{565} - 0.572\ (\varepsilon_{620}) + 0.246\ (\varepsilon_{650})] / 5.26 \quad (7)$$

$$\text{R-Phycocyanin (mg/mL)} = [\varepsilon_{620} - 0.666\ (\varepsilon_{650})] / 3.86 \quad (8)$$

$$\text{Allophycocyanin (mg/mL)} = [\varepsilon_{650} - 0.05\ (\varepsilon_{620})] / 4.65 \quad (9)$$

where ε is extinction coefficient, measured at 565, 620, and 650 nm.

Exo-polysaccharides separation. After separation of *Porphyridium purpureum* biomass by centrifugation, the aqueous solution containing exo-polysacharides and the remaining dissolved salts in the nutrient medium are concentrates through evaporation, at 80^0C up till 25% from the initial volume of 1:4. The resulting creamy greenish product was then mixed with alcohol (1:2 v/v), when raw exo-polysaccharides precipitate. Additionally, the precipitate was dried. The weighted exo-polysaccharides mass was characterized by thermal analysis using Thermogravimetric Analyser 951, Du Pont Instruments 2100 and by FTIR analysis (FTIR 6300 – Jasco provided with ATR Golden Gate – Diamond/Sapphire).

Lipid evaluation. Algal biomass is extracted in hexane using a Soxhlet – Lab-Line Multi-unit extraction heather; model Barn Stead/Lab-Line, followed by solvent removal on a rotary evaporator. Identification of lipids was made qualitatively by thermal analysis using Thermogravimetric Analyser 951, Du Pont Instruments 2100 and FTIR analysis (FTIR 6300 – Jasco provided with ATR Golden Gate – Diamond/Sapphire). Fatty acids composition, as methyl esters, was evaluated by GC - chromatography using the Perkin-Elmer Clarus 500 instrument.

Optimization experiment design. The experiments were focused on optimizing *Porphyridium purpureum* culture growth and its yields in value added green products. Basically, the aim of this investigation was to achieve higher

concentrations of phycobiliproteins (used as fluorescent pigments) in algal biomass and to increase the amount of exo-polysaccharides (used as biopolymers) in culture media. The first experimental series goal was the increasing of $NaHCO_3$ concentration in culture medium up to 3 g / L (the control experiment performed with the well known standard ASW growth medium, containing only 0.54 g $NaHCO_3$ /L) in order to raise both the algae rate of growth and growth process yield. Another target of the experiments was the accurate determination of the maximum $NaHCO_3$ concentration tolerated by *Porphyridium purpureum* culture. The second experimental series was set out for a study on the influence of light intensity (irradiation $\mu E/m^2s$ 120, and 240 $\mu E/m^2s$) on *Porphyridium purpureum* culture rate of growth and yields in useful specific components accumulated in the algal mass, under enriched culture medium (ASW culture medium supplemented with $NaHCO_3$).

3. Results and Discussion

Porphyridium purpureum growth process is clearly dependent on culture medium composition. Usual ASW culture medium proved to be a very convenient support in many algae species growth and production. The enrichment of this medium with CO_2, as a way for transferring this polluting product from the environmental atmosphere to some raw chemicals originating from algal mass, is certainly a route for furnishing photosynthesis carbon dioxide from the liquid phase at a higher rate than in the case of natural CO_2 absorption from air. According to the experimental set up, CO_2 carrier is $NaHCO_3$ provided as a supplement in the ASW culture medium. Experimental data on *Porphyridium purpureum* growth kinetics have been processed in careful agreement with Wood model illustrated in fig. 1 [17]. Fig. 2 displays the variation of the exponential growth rate and the content of the supplementary $NaHCO_3$ in the culture medium at an irradiance level of 240 $\mu E/m^2s$. Maximum growth rate was recorded at the 2g $NaHCO_3$/L concentration in culture medium. This concentration has to be considered as the upper admitted level of $NaHCO_3$ content. The results trend presumes a definite decay in growth rates at additional increases in the $NaHCO_3$ concentration. The same conclusion comes out from the computed doubling times t_D (Fig. 3). The minimum doubling time recorded at the 2g $NaHCO_3$/L concentration in culture medium also stands for the maximum production. When comparing with the production level assisted by the ASW culture media, the top exponential rate provides a theoretical 25.6% increase in total algal mass production.

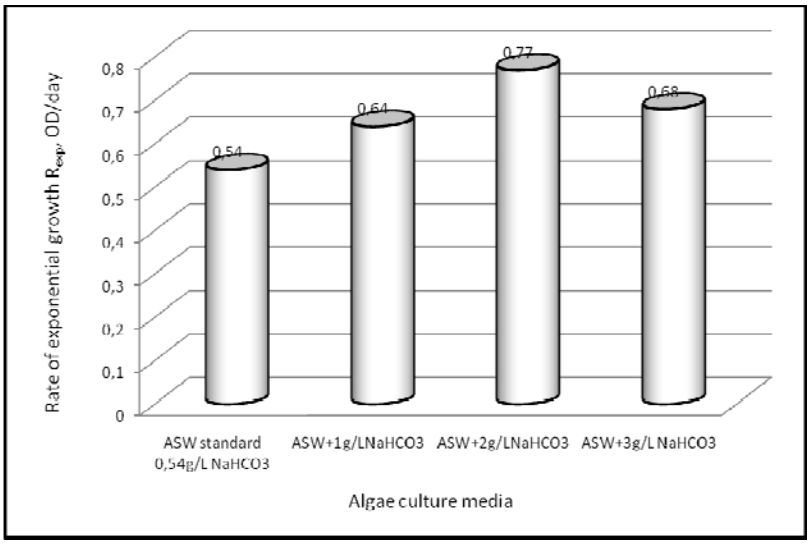

Fig.2. Algae exponential growth rates (R_{exp}) in the ASW and $NaHCO_3$ - enriched ASW culture media at 240 μE/m^{2}s irradiance

The light intensity plays an important role on *Porphyridium purpureum* culture growth. This means the raise of the irradiance from 120 μE/m^{2}s to 240 μE/m^{2}s results in higher growth rates both in standard culture medium (ASW control) and in nutrient medium supplemented with 2 g / L $NaHCO_3$ (fig.4). At E240 μE/m^{2}s, exponential growth rates over 0.75 days^{-1} were recorded for supplemented $NaHCO_3$ sample, i.g. over 25% higher then in the case of standard ASW medium. Consequently a significant drop in the doubling time (0.9 days) was recorded for 2 g/l $NaHCO_3$ added in ASW medium (fig.5). Figs. 6 and 7 are exhibiting data concerning the effect of irradiance level on the biomass production, as well as on the exo-polyssacharides yields.

As expected from the analysis of exponential growth rates, doubling the irradiance level has led to 25% higher yields in algal mass. Also, the irradiance proves to be a determining factor in the biosynthesis and accumulation of exo-polysaccharides. Thus, the *Porphyridium purpureum* growth under 240 μE/m^{2}s irradiance produces in the supplemented $NaHCO_3$ medium two times more exo-polysaccharides than in standard ASW medium at the same irradiance and 4.5 times more than in standard ASW medium at 120 μE/m^{2}s irradiance.

The content in each of the phycobiliproteins in Porphyridium purpureum culture growth under 120 and respectively, 240 μE/m^{2}s light irradiance, is presented in Fig. 8, as it was computed from the equations 7-9 and expressed in g/100 dry algal biomass.

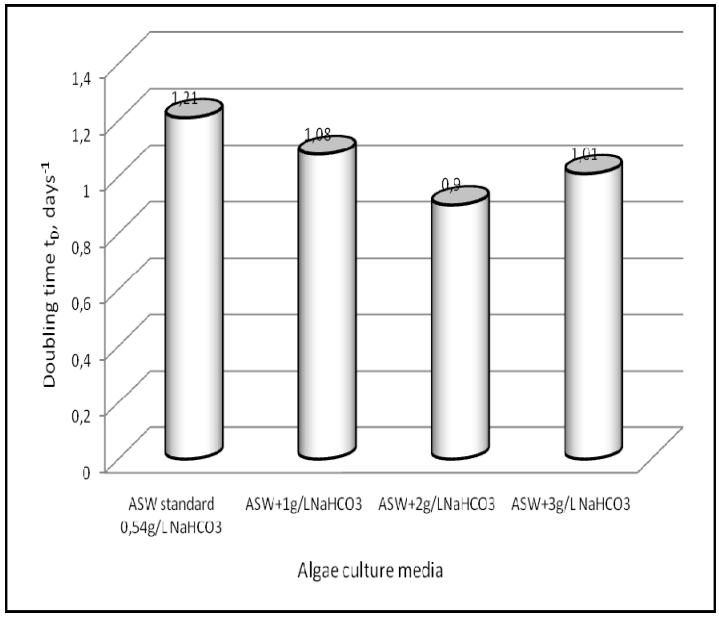

Fig.3. Algae doubling time t_D in the ASW and $NaHCO_3$ - enriched ASW culture media at 240 $\mu E/m^2s$ irradiance

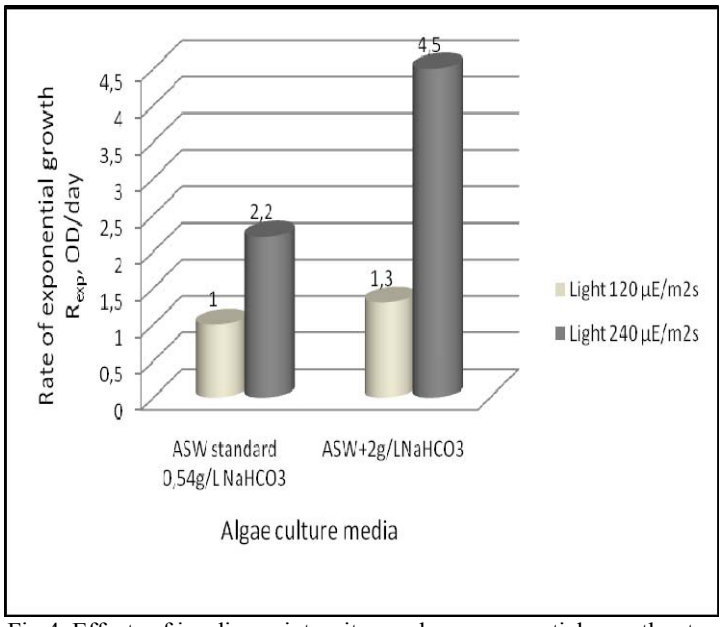

Fig.4. Effects of irradiance intensity on algae exponential growth rates

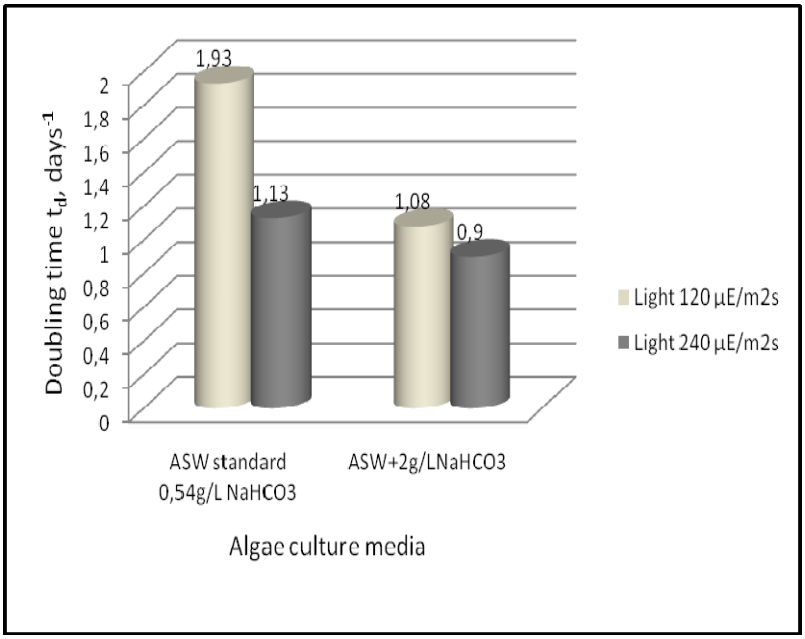

Fig 5. Effects of irradiance intensity on the algae doubling time t_D

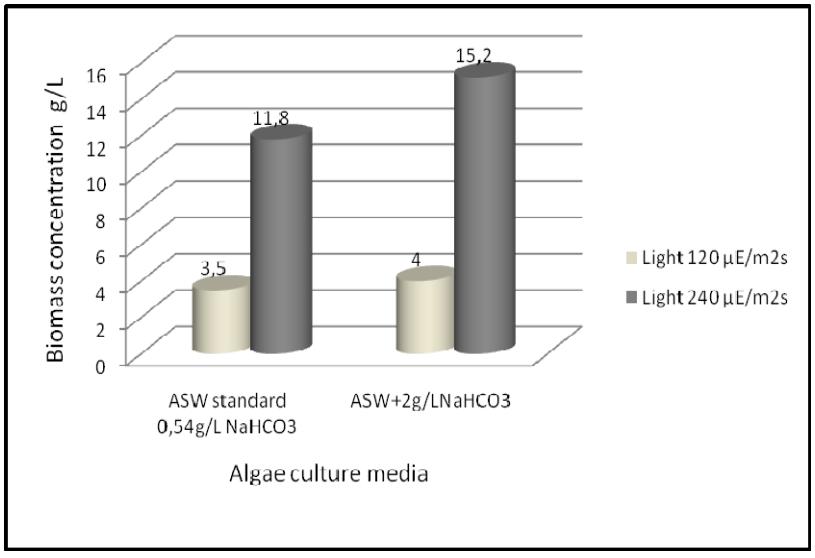

Fig 6. Biomass concentration from *Porphyridium purpureum* as function of light intensity

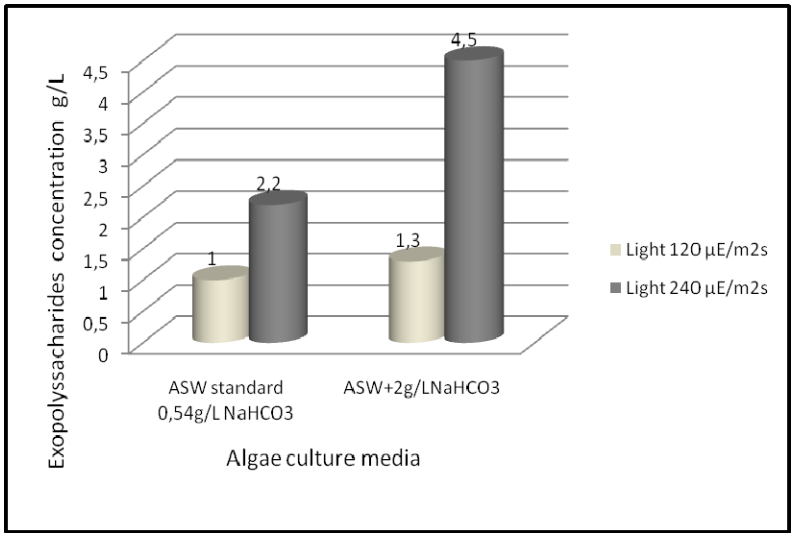

Fig 7. Exo polyssacharide concentration from *Porphyridium purpureum* versus the light intensity

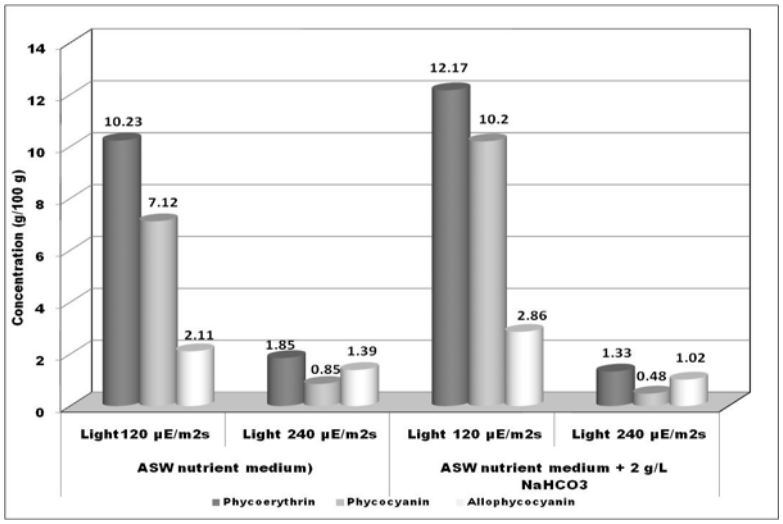

Fig 8. Content of individual phycobiliproteins in algal biomass

Lower light intensity and higher $NaHCO_3$ concentration in culture medium have a direct influence on phycobiliprotein accumulation in algal biomass. The maximum contents of each individual phycobiliprotein, expressed as g/100g dry algal biomass, are standing in the following range: Phycoerythrin 12.17, R-Phycocyanin 10.2, and Allophycocyanin 2.86 %.

Our experimental results are in agreement with Wang [2] data, who reported a biomass concentration of 3.27 g/L with a content of 132 mg/L phycoerythrin and respectively, the polyssacharides production of 543.1 mg/L. Also, Kathiresan [13] has obtained a maximum 4.8% phycobiliprotein yield in biomass and 3.3% phycoerythrin in algal dry mass at an irradiance of 18,85 $\mu E/m^2s$. Concerning the extracellular polyssacharide production of red microalga *Porphyridium* in an outdoor mass culture using a flat plate glass reactor, Singh [19] reported a value of 4.15 $g/m^2 \cdot day$.

Several approaches were used for analysis of algal biomass content in proteins, carbohydrates and lipids and exo-polysaccharides and lipids. Mainly, the dry algal biomass of *Porphyridium purpureum* cumulates 28% proteins, 24% carbohydrates and 11% lipids. Similar compositions of the dry mass were reported by Becker [1].

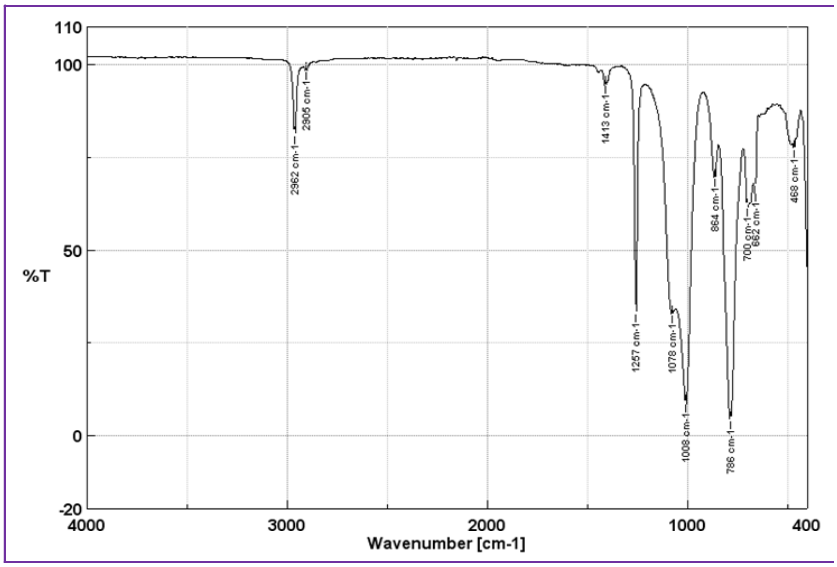

Fig 10. FT-IR analysis of *Porphyridium purpureum* lipids extract

The main classes of compounds from algal biomass (proteins, lipids and carbohydrates) were identified by means of FT-IR [20], due to their absorbance in different frequency regions in the mid-infrared part of the spectrum (fig.10). The following lines in FT-IR spectra accounted for the above biosynthesis products: 1016, 1075 and 1148 cm^{-1} - $\nu_{(C\text{-}O\text{-}C)}$ (stretching) in polysaccharides; 1246 cm^{-1} - $\nu_{(>P=O)}$ (stretching) in phophodiester of nucleic acids; 1370 cm^{-1} - $\nu_{(C\text{-}O)}$ (stretching) of COO^- groups in carboxylic acids from proteins; 1415 cm^{-1} - $\nu_{(CH2)}$ (bending) in proteins and lipids; 1543 cm^{-1} - $\nu_{(N\text{-}H)}$ (bending) in protein amides; 1638 cm^{-1} - $\nu_{(C=O)\ )}$ (stretching) in protein amides; 2918 cm^{-1} - $\nu_{(CH2)}$

(bending) in lipidic methylenes; 3289 cm^{-1} - water in carbohydrates. Also, the recorded lines 1026 and 1121 cm^{-1} - $\nu_{(C-O)}$ (stretching) in alcohols and respectively, ethers were reasonable considered as representative for exo-polysaccharides.

The total lipids content in algal biomass of *Porphyridium purpureum* by Soxhlet extraction in hexane ranges from 5 to 8%. Some specific FT-IR bands for lipid type compounds can be recognized in the Fig. 10.

The individual fatty acids, as methyl esters, from lipids extract were evaluated by gas chromatographic analysis (fig.11) as follows: C16 (26.92); C 16:1 trans (2.36); C 16:1 cis (1.61); C 18 (10.70); C 18: 1 cis (9.16); C 18: 2 cis (6.64); C 18: 3n6 (0.74); C 18: 3n3 (1.49); C 20: 4n6 (12.8); C 20: 5n3 (25.4); C 24:1 (2.18), (% on total fatty acids).

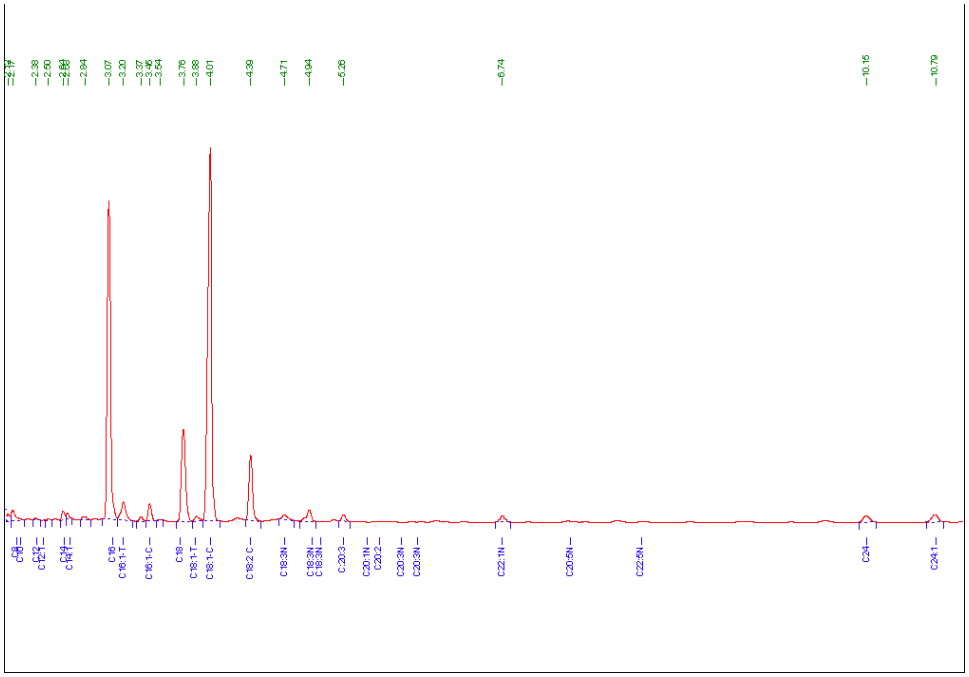

Fig 11. GC analysis of *Porphyridium purpureum* fatty acids in lipids extract

Most of the above identified compounds were found by Yongmanitchai and Ward [21] in their lipids extract from *Porphyridium purpureum.*

4. Conclusions

It was experimentally demonstrated the *Porphyridium purpureum* growth process follow the exponential kinetic model and the experimental data are in good agreement with the theory of phytoplankton population growth in laboratory batch photo-bioreactor. Also, the growth experiments have substantiated the two variables (light and sodium bicarbonate concentration) experimental design was a meaningful choice for the optimization of algal growth process parameters, as well as for predicting the phycobiliprotein/ exo-polysaccharide ratios in the dry algal biomass.

Reliable rates of growth were obtained for the growth process of *Porphyridium purpureum* at its exponential stage. Accordingly, under the higher irradiance level (240 $\mu E/m^2s$), the minimum doubling time was recorded at the 2g $NaHCO_3$/L concentration in culture medium. Mainly, for this concentration the exponential rate jumps from 2.2 OD/day at 120 $\mu E/m^2s$ to 4.5 OD/day at 240 $\mu E/m^2s$. When comparing the results with the production level assisted by the ASW culture media, the exponential rate at 240 $\mu E/m^2s$ provides a theoretical increase in total algal mass production from 4.0 g/L up to 15.2 g/L.

Light irradiance is a determining factor in the biosynthesis and accumulation of both exo-polysaccharides and phycobiliproteins. Actually, the biosynthesis processes of these two bio-product classes are rather concurrent in consuming light energy. Exo-polysaccharides biosynthesis is preferentially upturned from 1.3 g/L at 120 $\mu E/m^2s$ to 4.5 g/L at 240 $\mu E/m^2s$, under the best ASW culture medium supplemented with 2g $NaHCO_3$/L. By the contrary, the biosynthesis and accumulation of phycobiliproteins is highly disrupted by any increase in light irradiance beyond 120 $\mu E/m^2s$. Four to ten times decrease in each phycobiliprotein compound were recorded, when light irradiance goes from 120 $\mu E/m^2s$ to 240 $\mu E/m^2s$. Sodium bicarbonate supplemented in the standard ASW culture medium changes significantly the ratios between phycobiliprotein main compounds (phycoerythrin, R-phycocyanin and allophycocyanin)

5. Acknowledgements

We are grateful to National Authority for Scientific Research (NASR) for providing funding for GREENGHG Project.

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