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INNOVATIVE APPROACH FOR CO_2 FIXATION IN NEW PHOTOBIOREACTOR DESIGN AS A PERSPECTIVE FRAMEWORK FOR MICROALGAL GREEN FACTORIES

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Abstract: The integral biorefinery concept was realized in a multifunctional laboratory created in SAIM-BAS (The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences) with a new photobioreactor (PBR) design. The laboratory includes two stages: inoculum stage (8 PBRs with 1 litre geometric volume) and production stage (4 PBRs with 3 litters geometric volume) where concurrent hypotheses were checked. Small-scale PBRs in the inoculum stage with external white and internal green light were investigated by applying *Scenedesmus obliquus* strain. The maximum biomass achieved was 4.316 g/L. The dichloromethane extracts obtained from this biomass synergistically potentiated the antibacterial activity of gentamicin against a pathogenic strain of the

bacterial species *Staphylococcus aureus* at low concentrations (50 mg/L) thereby decreasing the minimal inhibitory concentration of the antibiotic fourfold (from 0.5 to 0.125 mg/L). In conclusion, the proposed PBR design for the inoculum stage is promising for maximum utilization of biomass in order to obtain high-value products.

The estimated potential of the *S. obliquus* extract to potentiate the activity of gentamicin will be of practical use by the development of health-promoting nutrients in human or veterinary medicine. The creation of multifunctional laboratories in Bulgaria based on our experience in Brazil demonstrated the success of our engineering approach to study integrated systems with many interrelations.

INTRODUCTION

Human society faces climate change problems which in a global sense are related to the greenhouse effect (Tol 2018). Increasing of CO_2 emissions from industrial waste gases in the atmosphere up to 411 ppm in January 2017, according to the Mauna Loa Observatory, shows the scale of disasters, which we are going to face in the near future. Hence, the reduction of CO_2 emissions undoubtedly is a milestone for environmental protection (Pires et al. 2012, Cheah et al. 2016). Furthermore, recent developments of biological methods for CO_2 capturing including microalgae and cyanobacteria are crucial within the framework of a biorefinery approach and determine the engineering challenges (Kroumov et al. 2021a, Kroumov et al. 2021b). Scientists focus on their studies to isolate and use microalgae species tolerant to the high concentration of CO_2. The methods include application of CO_2 in the form of pure gaseous CO_2, real or simulated flue gas, or soluble carbonate (bicarbonate) which manifested itself also as an increase in biomass productivity (Singh et al. 2014, Kroumov et al. 2016, Aslam et al. 2017, Kuo et al. 2017). Detailed information can be found in elaborated reviews (Kroumov et al. 2016, Thomas et al. 2016, Vuppaladadiyam et al. 2018). The fate of the supplied carbon can end up in making a scaffold for lipids, proteins, sugars and pigments (Sydney et al. 2010). Despite such remarkable potential, the production of microalgae for low-value bulk products, such as proteins for food/feed applications, fatty acids for nutraceuticals, or bulk products such as biofuels, is heretofore, not economically feasible (Zhou et al. 2017). Recent techno-economic analyses and life-cycle assessments of microalgae-based production systems have suggested that the only possible way of realizing the potential production is to completely use the biomass in an integrated biorefinery set-up wherein every valuable metabolite is processed in a downstream stage – extracted, and valorized for different activities (Chew et al. 2017, Kroumov et al. 2017, Gonçalves et al. 2019a, Gonçalves et al. 2019b, Scheufele et al. 2019).

The efforts of the scientists working in the field of CO_2 sequestration by microalgae are directed towards minimizing the CO_2 emissions (Chagas et al.

2016, Olofintuyi et al. 2016, Thomas et al. 2016). Therefore "green technologies" using freshwater and marine microalgae have to be competitive on the market which relies on the maximization of biomass concentration in photobioreactors (PBRs) as well as production of high-value products (HVPs), pharmaceuticals (Borowitzka 1995, Yan et al. 2016) and bioactive compounds, such as antibacterial (Schuelter et al. 2019), antifungal (Najdenski et al. 2013) and anticancer metabolites, (Shanab et al. 2012, Martínez Andrade et al. 2018) among others.

The system analysis theory (Kroumov et al. 2016) used for our research approach in this investigation has been already successfully applied to study chemical, biochemical, biotechnological processes and (photo)bioreactors and further to model, optimize and design them (Kroumov et al. 2017, Gonçalves et al. 2019a, Gonçalves et al. 2019b, Scheufele et al. 2019, Schuelter et al. 2019). The decomposition principles of the theory are its milestones where the system (PBR, in this case) is divided into sub-systems. The application of the whole scheme to microalgae technology is possible as well (Fig. 1).

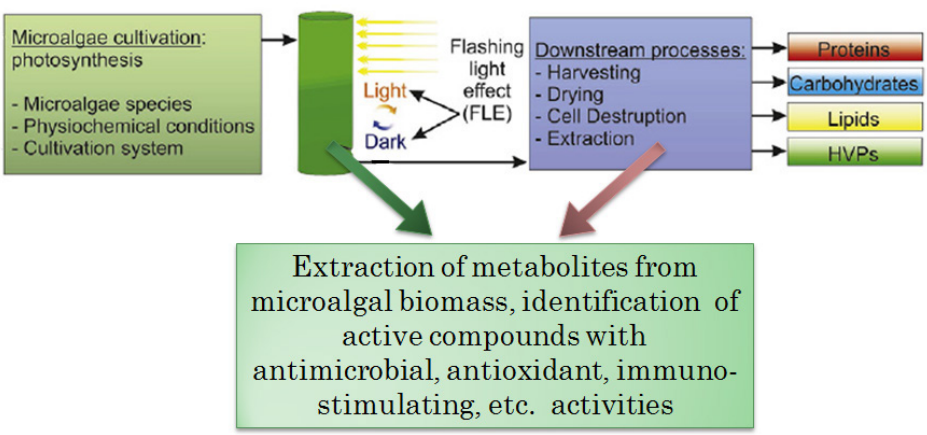

Figure 1. The scheme of microalgae “green technology”

The first key step is culturing the microalgae in PBRs. Our complex analysis of PBR as a system has received much international attention, recently (Sauer et al. 2021). Using PBR as a system is discussed, analyzed, and modeled below and the relationships between sub-systems are considered. A simplified scheme of the PBR as a system is shown in Fig. 2.

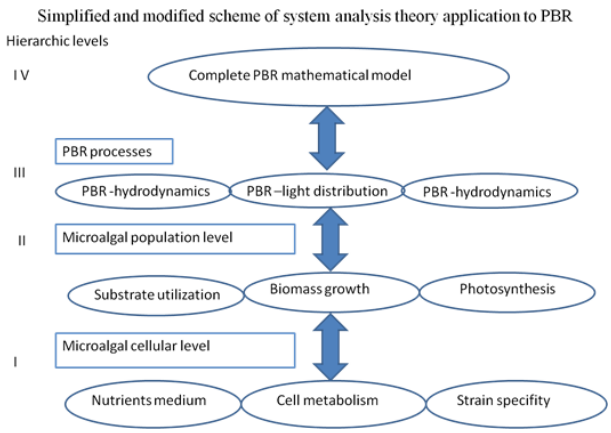

Figure 2. A simplified and modified scheme of the system analysis theory is applied to PBR as a system

In this scheme, we recognize four relatively separated hierarchic levels in order to study and model them easily. First, it is especially important to find out how to calculate the nutrient medium for autotrophic culturing of microalgae in order to maximize the cell concentration in the particular PBR design. We developed and published a complex theoretical approach for nutrient medium optimization. In deep details, this work can be found elsewhere (Kroumov et al. 2015, Kroumov et al. 2016). Having this method as a tool (see Fig.3), we were able to develop several processes in the USA, Bulgaria, and Brazil and to achieve excellent results (Hinterholz et al. 2019, Schuelter et al. 2019).

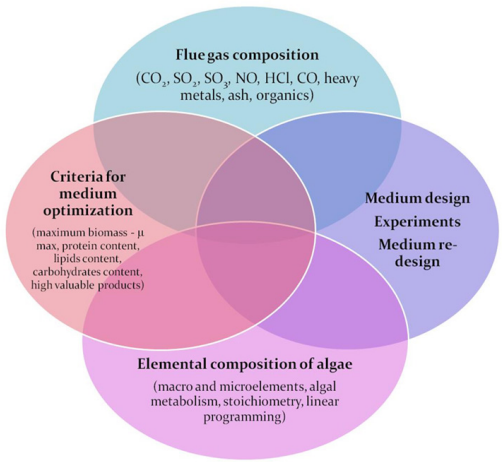

Figure 3. Adopted algorithm for medium optimization in connection to flue gas as a source of CO_2 for autotrophic growth of microalgae strains

The next step in process development is to understand how microalgal kinetics (photosynthesis) is linked to light availability in the PBRs. A model was developed where S/V ratio and specific growth rate were linked (Kroumov et al. 2013). The main conclusions from this work can be presented as follows:

-$S_f/V=4/D_{PBR}$, this simple equation shows that light availability depends only on tubular PBR's diameter.

-I_f D_{PBR}=0.01 m, then the ratio is equal to 400 m2/m3 which is the maximum for practical applications.

Based on the principles of the system analysis theory (Kroumov et al. 2016) and the integral biorefinery concept (Kroumov et al. 2021a), we set ourselves the goal to establish a multifunctional laboratory in Sofia and Plovdiv, Bulgaria, based on our previous experience in UNIOESTE – Toledo, PR Brazil (Hinterholz et al. 2017, Hinterholz et al. 2019, Schuelter et al. 2019). In the multifunctional laboratory in Bulgaria, several types of tubular and flat-plate PBRs were designed to fulfill a two-stage system for incubation and production phases (see Fig. 4).

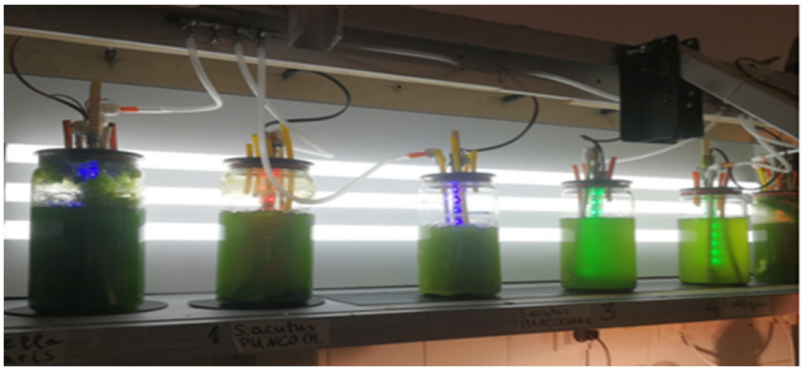

Figure 4. A multifunctional laboratory in Sofia – incubation stage,
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The laboratory potential offers complex studies in two steps – incubation and production stage based on investigation of different microalgal species in innovative small column PBRs (incubation stage, Fig.4) with internal illumination where various light waves and irradiation conditions can be applied. This approach gave us the opportunity to study different biological effects of extracts obtained from the microalgal species *Scenedesmus obliquus*. Some preliminary results on the antimicrobial activity of a dichloromethane extract obtained from biomass from a selected strain *S. obliquus* in the inoculum stage will be discussed.

MATERIALS AND METHODS

Algal strain and culture conditions

The algae strain *Scenedesmus obliquus* (Turpin) Kützing, strain 8610 used in this study was kindly provided by the National Algae Culture Collection (PACC, Plovdiv, Bulgaria) and stored on BBM agar medium according to Bischoft & Bold (Bischoff et al. 1963) in a luminostat chamber, under low illumination and at T=12°C. A modified M-8 medium was used for cultivation (Mandalam et al. 1998) in order to reach high-density culture (Kroumov et al. 2015). The medium composition (g/L) was as follows: 3.0 KNO_3; 0.74 KH_2PO_4; 0.013 $CaCl_2 \cdot 2H_2O$; 0.13 $FeSO_4 \cdot 7H_2O$; 0.4 $MgSO_4 \cdot 7H_2O$; 0.04 NaEDTA·Fe; and 0.26 $NaHPO_4$. Sodium bicarbonate served as a buffer (pH=7.4). The microalgae were cultured at room temperature of 24–28°C under external white and internal green light in small PBRs representing bubble columns with 8.5 cm inner diameter and 19 cm height. The temperature inside the PBRs was 28.8-37°C. The light intensity photon flux of the external light was achieved by using three 1200 mm LED lamps placed behind the PBRs: T8 SMD, 18 W 1800 lm 6400 K and AC 230V 50 Hz. A strong-light halogen lamp (approximately 500 W) was placed in front of the PBRs in order to stress the cells to synthesize biologically active compounds. The photoautotrophic growth occurred under the continuous light period of 24 h and a constant supply of 0.1–0.3/L/L/min air flow containing 2–10% CO_2. The pH ranged from 7.5 to 9.0 during the cultivation depending on the growth and CO_2 supply.

Dry weight estimation and calculation of specific growth rate

The dry weight (DW) was estimated based on samples of 50 mL withdrawn from the PBRs. The samples were centrifuged for 10 min at 4000× g, the supernatant was discarded and the pellet was dried at 105 °C until a constant weight was reached.

The model-based approach represents mass balances of key components in every level combining all the knowledge in the system of non-linear ordinary differential equations (ODE). The growth rate was calculated for the exponential phase according to equation (1):

$$\mu = \frac{(\ln(X2) - \ln(X1)}{(t(2) - t(1))}; \left[\frac{1}{d}\right] \quad (1)$$

Note: X(2) and X(1) are the biomass concentrations measured in time t(2) and t(1).

The program for calculation was coded in MAPLE® -15 symbolic mathematic software. ODE was solved by using "dsolve, Stiffness", "dsolve/numeric/lsode" packages.

Lyophilization of microalgal biomass and preparation of dichloromethane extracts

At the end of the cultivation process (after entering the stationary stage) the microalgal biomass was harvested after centrifugation (20 min, 4000× g) and freeze-dried (lyophilized) in a vertical freeze dryer (BIOBASE Group, BK-FD18P, Jinan, Shandong, China). Briefly, the microalgal biomass was frozen within the lyophilizer at −80°C for 4 hours. Thereafter, the samples were placed on shelves in a standard chamber under a deep vacuum and left for 48 h until full water evaporation and obtaining of lyophilized biomass.

A dichloromethane extract was prepared from 2.2157 g of the lyophilized microalgal biomass as published before (Zaharieva et al. 2022). Briefly, an ultrasound-assisted extraction was applied (three times in 100 mL of dichloromethane for 15 min each time) to yield 0.1052 g (4.75%) of extract. The working solution of the extract (50 mg/mL) for estimation of antimicrobial activity was prepared in ethanol (96%, #603-002-00-5, Honeywell Specialty Chemicals, Seelze, Germany) under ultrasonication (ultra-sound bath BIOBASE Group UC-20C, Jinan, Shandong, China).

Evaluation of antimicrobial activity

The antimicrobial activity of the dichloromethane extract obtained from the microalgae strain *S. obliquus* 8610, after cultivation in a small-scale bioreactor under white (external) and green (internal) light was evaluated via the broth microdilution method (BMD) according to ISO 20776/1 [ISO20776/1 2006]. The reference strain Staphylococcus aureus ATCC® 29213™ (American Type Cell Culture Collection, USA) was used as in vitro model. The minimal inhibitory concentration (MIC) of gentamicin was evaluated according to the recommendations of EUCAST (EUCAST 2021).

The combined effect was determined using the checkerboard assay (Jenkins et al. 2012). The extract was combined with gentamicin. Briefly, serial dilutions of the extract and the antibiotic gentamicin were prepared in a two-dimensional fashion to include 42 combinations in a 96-well plate. Clinically relevant concentrations of gentamicin were used (0.03125-2 mg/L). The combination effects were calculated following the fractional inhibitory concentration (FIC) methodology as described before (Jenkins et al. 2012, Zaharieva et al. 2019, Zaharieva et al. 2022).

The FICs were calculated and interpreted as follows:

•Step 1

$$FIC\ (A) = \frac{MIC_C\ (A)}{MIC\ (A)} \quad (2)$$

$$FIC\ (B) = \frac{MIC_C\ (B)}{MIC\ (B)}, \quad (3)$$

Where FIC stands for fractional inhibitory concentration, A stands for drug A (penicillin) and B stands for the microalgae extract.

•Step 2

$$\sum FIC = FIC\ (A) + FIC(B) \quad (4)$$

Synergy is defined as ΣFIC≤0.5, indifference – as 0.5<ΣFIC≤4 and antagonism – as ΣFIC>4. Some investigators [20] consider compounds additive when 0.5<ΣFIC≤1.

RESULTS

The growth rate of the microalgae strain *S. obliquus* 8610 in small-scale PBRs

The results from the growth rate of the chosen strain *S. obliquus* 8610 are presented in **Table 1.** For the calculation of the growth rate, we chose two points within the exponential growth phase of the microalgae at an interval of 15 days.

Table 1. Biomass dry weight is used for calculation of the specific growth rate

Sample	Date of the sample collection	Biomass [g/L]
S. obliquus	10.6.2021 (day 14 of the cultivation)	3.36
S. obliquus	25.6.2021 (day 29 of the cultivation)	6.95

Equation (1) was applied to calculate the specific growth rate of the microalgal strain under the described conditions and the results were:

•μ(SC-PBR1)= 0.048 d^{-1} – SGR (specific growth rate during logarithmic growth);

•X(2)= 4.32 [g/L]; t(2) = 29; X(1) = 2.85 [g/L]; t(1)= 14 [d]; time interval 15 days.

It has to be noticed, that the data from the incubation stage are mostly for checking different hypotheses for photosynthesis under different working conditions and nutrients.

Antimicrobial activity of S. obliquus dichloromethane extract on *S. aureus*

The estimated minimal inhibitory concentration after a single application of the extract on the chosen S. aureus strain was higher than 12.5 mg/mL. However, low concentrations of the microalgae extract (0.05 - 0.2 mg/mL) decreased two-fold the active concentration of the antibiotic gentamicin and led to a synergistic effect. The results from the combination and the single effects are presented in Table 2.

Table 2. The anti-staphylococcal activity of dichloromethane extract from *S. obliquus* subs. oblongata cultivated in a small-scale bioreactor under white (external) and green (internal) light

MIC of *S. obliquus*, dichloromethane extract	**MIC of gentamicin**	**FIC of A**	**FIC of B**	**∑FIC**	**Effect**
> 12.5 mg/mL	0.5 mg/L	0.125	0.05	0.30	Synergism

DISCUSSION

This work highlighted the potential of the established two-stage PBR system for maximal biomass utilization in terms of using biomass from the inoculum stage for extracts with antimicrobial activity under the given conditions. The chosen strain *S. obliquus* achieved high biomass concentration in an innovative small PBR type under the supply of different white external and green internal light irradiation. In a preliminary study, the photostimulation of the intracellular synthesis of carotenoids was expressed under conditions of low energy 85 μmol m^{-2} s^{-1} for the given time of cultivation (Gonçalves et al. 2019b). In some experiments, a higher energy irradiation of 595 μmol m^{-2} s^{-1} suggests the photoinhibition of the microalgae because it did not stimulate the synthesis of carotenoids and the growth did not increase. Hence, the limits of the light intensity change always have to be defined and taken into account in order to avoid non-optimal conditions of cultivation. Therefore, the experimental conditions in our study were directed in low energy illumination below 85 μmol m^{-2} s^{-1}.

Specific growth rate (SGR) as a key parameter of microalgae growth was measured under logarithmic growth and its value of μ(SC-PBR1)= 0.038 d−1, time= 15 d can be considered under such conditions (pH, T, mixing and PBR design) as sufficiently good for achieving the set goal – to obtain biomass with antibacterial potential within the inoculum stage. Improvement of the SGR definitely will be achieved when the culture will be transferred in the production stage where the "flashing light effect" and fluid flow distribution can be controlled and optimized, which warrants further experiments.

Analysis of the presented data about the anti-staphylococcal activity of the investigated extract leads to the conclusion that the single application is characterized by a weak antibacterial effect while the synergistic combination with gentamicin is perspective and shows the potential of such microalgal extracts for combined application with clinically used antibiotics aiming at increasing the efficacy of the antibiotic at lower doses. According to published data, the antibacterial activity of Scenedesmus spp. extracts differs depending on the bacterial strain and the solvent used for extraction, and generally, the effect against Gram-positive bacteria was found to be higher than against Gram-negative bacteria (Najdenski et al. 2013, Vidyashankar et al. 2014, Marrez et al. 2019, Austic et al. 2021, El-Baz et al. 2021). Thus our results confirm the published data. However, there are no data about the potential of *S. obliquus* extracts to increase the activity of clinically used antibiotics except for recent investigation (Zaharieva et al. 2022). Therefore the data about the synergistic effects of the dichloromethane extract with gentamicin evaluated in this study represent the first such report. As published before, the chemical analysis of the biomass revealed that the combination green (internal): white(external) light promoted the accumulation of 5.5 mg/g DW carotenoids and 8.9 mg/g DW lipids (Zaharieva et al. 2022). The MS/MS spectrum performed in the same study confirmed the presence of unsaturated fatty acids and the carotenoids lutein/zeaxanthin, violaxanthin, carotene and others. Fatty acids with more than 10 carbon atoms have been well known for their ability to induce lysis of bacteria (de Morais et al. 2015). The presence of several long-chain fatty acids in our extract (Zaharieva et al. 2022) would explain the synergistic effect with gentamicin. It could be hypothesized that the extract increases the penetration of the antibiotic into the bacterial cells but further studies are needed to elucidate this possible mechanism of action. The presence of carotenoids in our extract suggests not only antioxidant but also antibacterial potential. As far as carotenoids extracted from different microalgal and plant sources have been found to exert antibacterial activity (Ambrico et al. 2020, Karpiński and Adamczak 2019), the presence of the above-mentioned carotenoids most probably also contributes to the observed antibacterial activity of our extract in combination with gentamicin. Further studies are needed to evaluate in detail the antioxidant potential of the obtained dichloromethane extract which has been shown to have radical-scavenging activity as published in (Zaharieva et al. 2022).

CONCLUSION

This work presents the methodology of system analysis theory, which our group used in the creation of multifunctional laboratories in Sofia and Plovdiv in two stages – incubation and production. Combining the theory with the integral biorefinery concept, we were able to set up the different types of PBRs in the

inoculation and production stages of the multifunctional laboratory. Further, the dichloromethane extracts obtained from *S. obliquus* biomass under green (internal): white (external) low light illumination was analyzed. It was found that there is a synergism between extracts at low concentrations (50 mg/L) and gentamicin. Antibacterial activity of a combination of these compounds against a pathogenic strain of the bacterial species *S. aureus* was detected, thereby diminishing fourfold (from 0.5 to 0.125 mg/L) the minimal inhibitory concentration of the antibiotic. Maximization of biomass concentration (4.32 g/L) in the PBRs in the inoculum stage was achieved. Hence, experiments on antibacterial and antioxidant activities of extracts obtained from the microalgal species *S. obliquus* were very promising. Moreover, our experiments in such multifunctional laboratories proved the concept that such laboratories are innovative, very promising, and have a bright future.

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Conflicts of Interest: The authors declare no conflict of interest.

Author contribution statement: A.K. and H.N. planned and designed the experiments. T.C.K., M.M.Z., Y.I., A.B., S.R.-V., A.A., D.Z.-D., V.B., R.G., M.K., M.M. and A.G. performed the experiments. A.K., H.N., M.M.Z., T.C.K. and Y.I. analyzed the data. A.K., M.M.Z., T.C.K., M.M. and Y.I. and wrote the manuscript.

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