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MULTIFUNCTIONAL ALGOLOGY LAB FOR CO_2 FIXATION BY *SCENEDESMUS OBLIQUUS* WITH POTENTIAL FOR SYNTHESIS OF HIGH-VALUE PRODUCTS

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Abstract: Several different types of photobioreactors (PBRs) were designed in order to improve the selection of microalgal strains of freshwater algae isolated from Bulgarian locations. The criteria for strain selection are based on their potential for a high specific growth rate and proven ability to synthesize biologically active metabolites with antimicrobial activity. Experimental sets of batch processes were performed with the promising *Scenedesmus obliquus* 8610 strain cordially provided by National Algae Culture Collection-Plovdiv (PACC). The preliminary experiments and results on the antimicrobial activity of the extracts from *S. obliquus* biomass were promising. By calculating the nutrients of the medium using a linear programming procedure and by applying the M-8 modified medium recipe, we were able to achieve maximum biomass concentration in the flat plate-PBRs under given working conditions. The strategy to find new valuable metabolites from this strain passed through the manipulation of various state parameters and stress factors. We believe that by completing this research, we will be able to offer a robust strain and a technological protocol for its subsequent industrial application.

INTRODUCTION

The modeling of cultivation systems is accepted as a basic rule in the production of algae crops. Its contribution can save resources and make processes profitable. Algae cultures have high promise as sources of biologically active compounds (BAC) (Schuelter et al., 2019). Microalgae can become an ecologically clean and economically viable source of compounds of interest, as production can be optimized under controlled conditions (Kroumov et al., 2016). BAC derived from microalgae have anti-inflammatory, antimicrobial, and antioxidant properties. In addition, these organisms had the ability to strengthen health and reduce the risk of developing degenerative diseases (De Morais, 2015). The properties of green microalgae to accumulate large amounts of biomass have been extensively reported for opportunities to produce oil-based biodiesel as well as biosorbents (Makareviciene et al., 2011; Chellamboli et al., 2014; Marcou et al, 2015; Ge et al, 2016). In some cases, a substitute for various feeds and functional foods was sought, which at the same time had a content richer in essential amino acids than plants (Patnik et al,2019). In search of unconventional sources rich in biologically active substances, the focus of scientists is on algae, which are reservoirs of proteins, lipids, carbohydrates, vitamins and pigments (Patnaik et al, 2019). Many reports have described the diversity of accumulated biological substances in algae, paying attention to their biological actions.

Antimicrobial activity of various algal cultures has been found in many species and is due to the ability to synthesize compounds such as fatty acids, acrylic acids, halogenated aliphatic compounds, terpenoids, sterols containing sulfur heterocyclic compounds, carbohydrates, acetogens and phenols. Some of them have been shown to have the potential to produce compounds such as α- and β-ionone, β-cyclocitral, neophytadiene and phytol (Prakash et al., 2011). Antimicrobial activities against human pathogens, such as *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Staphylococcus epidermidis*, have been attributed to γ-linolenic acid, eicosapentaenoic acid, hexadecatrienolic acid, docosahexaenoic acid, isolated from microalgae (Amaro et al., 2011; De Morais, 2015).

The aim of the present study was to determine the capacity of *Scenedesmus obliquus* for cell growth in photobioreactor culture systems including inoculum and production stages as well as the antimicrobial activity of the extracts.

MATERIAL AND METHODS

Algae strain

Algae strain Scenedesmus obliquus 8610, part of the National Algae Culture Collection-Plovdiv (PACC) was used (Fig. 1). The strain was isolated from Rupite, Blagoevgrad region (Bulgaria) and stored on a laboratory prepared BBM agar medium according to Bischoft & Bold (1963).

Medium and culture conditions:

The modified cultural medium (Hinterholz et al., 2019) has the following composition: (mg/L): Urea 300; $(NH_4)_2SO_4$ 660; NH_4NO_3 400; KH_2PO_4 740; $CaCl_2.2H_2O$ 13; $FeSO_4.7H_2O$ 130; $MgSO_4.7H_2O$ 400; $MnCl_2.4H_2O$ 12.98; $CuSO_4.5H_2O$ 1.83; $ZnSO_4.7H_2O$ 3.2. The pH 7.5 of the medium was maintained throughout the process. The temperature was chosen as optimal for growth, 30°C±1°C (Hinterholz et al., 2019).

Installations:

Inoculum stage: The inoculum was prepared in glass cylinders used as column photobioreactors (PBRs, in figure 2 below). The working volume was V=0.5 L. The culture was transferred to a flat plate PBRs (FP-PBRs) (Fig. 2 above) with a working volume of V=1.5 L. The cultivation process was carried out in batch mode. Behind the PBRs 6 LED tubes LT with 9W were installed. A temperature of 30°C±1°C was maintained in the dynamics of the process. The process parameters were monitored for 33 days. The CO_2 consumption was calculated by YCO2/X, which for algae is approximately 1.8 g CO_2 to form 1 g algae biomass.

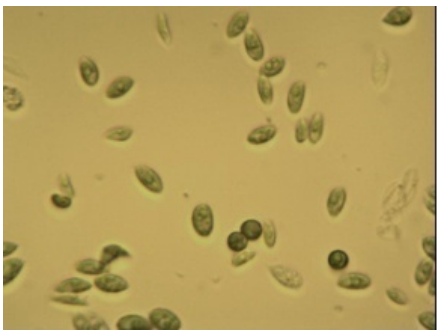

Figure 1.
Scenedesmus obliquus 8610 (PACC)

Figure 2.
PBRs in inoculum (bellow) and production stage (above)

Analytical methods:

In the dynamic of cultivation, the biomass was separated from the cultural liquid by centrifugation at 5000×g for 20 min. The dry biomass was determined by the weight method after drying to constant mass at 105°C.

The specific growth rate was calculated on the basis of the following mathematical equation:

$$\mu = \frac{(\ln(X(1)) - ln\,(X(0))}{t(1) - t(0)}$$

where, X(1) and X(0) stands for the biomass concentrations at the time of cultivation t(1) and t(0) for the selected time interval; μ -stands for the specific growth phase. Time can be chosen for any part of the growth curve.

In order to preserve the biologically active substances in the algal biomass, we used a gentle method of lyophilization. The dried biomass samples were used for sample preparation and antimicrobial activity.

To obtain the extracts freeze-dried biomass dissolved in methanol and ultrasonic extraction by the method of Dagnon et al. (2019) were used.

Bacterial Strains and Culture Mediums

The following bacterial strains were used for the determination of minimal inhibitory concentrations (MICs): *Staphylococcus aureus* (ATCC® 29213™, American Type Cell Culture Collection, Manassas, Virginia, USA), *Staphylococcus aureus*—MRSA (NBIMCC 8327—resistant to methicillin and oxacillin, National Bulgarian Collection for Industrial Microorganisms and Cell Cultures), *Escherichia coli* (ATCC® 35218™, Manassas, Virginia, USA) and *Salmonella typhimurium* (Strain 123, Collection of the Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences). All strains were grown in Mueller Hinton broth (MHB, #M0405B, Thermo Scientific-Oxoid, Hampshire, UK) and agar (MHA, #CM0337, Thermo Scientific-Oxoid, Hampshire, UK).

Determination of minimal inhibitory concentrations (MIC)

The minimal inhibitory concentrations were determined with the broth microdilution method (BMD) according to ISO 20776-1:2006 (I*SO/TC 212. ISO20776/1-2006*). Twofold serial dilutions of the extract ranging from 0.078 up to 10 mg/mL were prepared in 96-well plates (50 μL/well) in a triplicate. An equivalent volume of the bacterial suspension (1×10^5 CFU/mL) was added to each well. The plates were incubated at 37 °C for 24 h. The lowest drug concentration, which inhibited the bacterial growth was accepted as MIC. Gentamicin (0.008–4 mg/L), penicillin (0.008–4 mg/L) and ciprofloxacin (0.005-1 mg/L) were used as

the reference antibiotics (positive control). The recommendations of EUCAST (European Committee on Antimicrobial Susceptibility Testing) were followed for the analysis of the results (*EUCAST, 2021*). MHB served as a negative control.

RESULTS

Experimental sets of batch processes were performed with the promising *Scenedesmus obliquus* 8610 strain in flat plate innovative (FP-PBRs). *S. obliquus* had growth characteristics which we reported on day 27 from the beginning of the cultivation process. The specific growth rate achieved in FP-PBRs during the cultivation of *S. obliquus* in the production stage (Fig.2 above, Fig. 3) under the working conditions was $\mu = 0.051\ d^{-1}$. The growth of this strain can be much faster when a higher CO_2 content than the atmospheric value is supplied in both types of PBRs in the scheme. The cell growth was monitored under a microscope. A correction on pH was made several times in the time of the cultivation process. At a working volume of 1.5 L, an absolute dry weight of 2.8 g/L was reached on day 27 in a logarithmic growth phase (Fig.2 above, Fig.3).

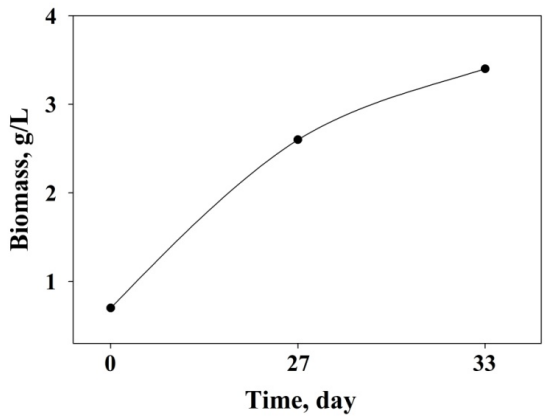

Figure 3 Time course of biomass accumulation by S. obliquus in flat plate innovative photobioreactor cultivation system.

The methanol extract obtained from *S. obliquus s*howed antibacterial activity against the Gram-positive bacterial species Staphylococcus aureus. Two *S. aureus* strains were used in our study – one penicillin and methicillin-sensitive strain and one methicillin-resistant strain. The MIC for both strains was 5 mg/mL (Table 1). The extract did not exhibit any activity against the Gram-negative strains E. coli and *S. typhimurium.*

Table 1 Antimicrobial activity of a methanol extract of *S. obliquus*

Bacterial strain	*S. obliquus* **extract concentration [mg/mL]**	**Reference antibiotics (MICs)**
Staphylococcus aureus ATCC 29213	5	Penicillin 0.125 mg/L
MRSA NBIMCC 8327	5	Gentamycin 0.25 mg/L
Escherichia coli ATCC 35218	-	Ciprofloxacin 0.0125 mg/L
Salmonella typhimurium 123	-	Ciprofloxacin 0.05 mg/L

Legend: MICs – minimal inhibitory concentrations.

DISCUSSION

S. obliquus was used as a producer of biologically active substances by other authors (Wang et al., 2019; El -Chaghaby, 2019; Ibrahim, 2021). Our aim was to show the capacity of a strain isolated from Bulgarian locations for cell growth in photobioreactor systems and the antibacterial potential of extract from the cultivated biomass. The selection of strains with high biomass productivity and their adaptation to laboratory conditions are two important key characteristics for successful algal cultivation. Our results for biomass accumulation by *S. obliquus* were similar to the ones reported by authors (Duan et al., 2019).

By calculating the nutrients of the medium using a linear programming procedure and elemental analysis of the cells we applied the M-8 modified medium recipe (Kroumov et al., 2015). Further studies will show the great potential of the strain in the created innovative PBRs (Fig. 2, above). We were able to achieve maximum biomass concentration in the flat plate-PBRs under given working conditions.

The extracts of Scenedesmus spp. have been often described as antimicrobial agents against foodborne pathogens such as *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Salmonella* sp (Elfata et al., 2017). An important relationship was observed between the type of microalgal extract of the species Scenedesmus and the strength of the antimicrobial action. Diethyl extract had an inhibitory effect against *S. typhi* and *P. aeruginosa*, as well as against *Bacillus cereus, S. aureus, E. coli* and *Klebsiella pneumoniae*, while chloroform, ethyl acetate and hexane extracts showed weaker effects (Marrez

et a.,2019). In our previous studies on dichloromethane extracts obtained from *S. obliquus,* we reported significant antimicrobial activity on *S. aureus, E. coli* and *S. typhimurium* (Zaharieva et al., 2022). The total methanol extract of the same strain in this study showed moderate antibacterial activity against Gram-positive bacterial strains which is indicative of the capacity of this strain to be further explored aiming at the targeted production of antimicrobial metabolites. It is worthy to investigate the chemical composition of the biomass in future studies in order to extract fractions with the potential for antibacterial activity.

CONCLUSION

The strategy to find new valuable metabolites from this strain passes through the modulation of various state parameters and stress factors. We believe that by completing this research, we will be able to offer a robust strain and a technological protocol for its subsequent industrial application.

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

Author Contributions: A.K. and H.N. planned and designed the experiments. S.R.-V., T.K., M.M.Z., Y.I., A.B., 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., S.R.-V., T.K. and Y.I. analyzed the data. A.K., M.M.Z., T.K., M.M., S. R.-V. and Y.I. and wrote the manuscript.

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