Հայաստանի Գիտությունների Ազգային Ակադեմիա
Национальная Академия Наук Армении
National Academy of Sciences of Armenia

Հայաստանի Կենսաբանական Հանդես
Биологический Журнал Армении
Biological Journal of Armenia

• Փորձարարական և տեսական հոդվածներ • Экспериментальные и теоретические статьи •
• Experimental and theoretical articles •

Biolog. Journal of Armenia, 4 (72), 2020

COMPARATIVE ASSESSMENT OF BREWERY WASTEWATER TREATMENT POTENTIAL BY MICROALGAE *PARACHLORELLA KESSLERI* AND *CHLORELLA VULGARIS*

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The possibility of brewery wastewater treatment by *Parachlorella kessleri* and *Chlorella vulgaris* microalgae strains and the efficiency of wastewater treatment depending on different concentrations of inoculum volume have been studied. The prospects of using brewery wastewater as a substrate for fatty acids production were also investigated in the framework of this research.

Microalgae – brewery wastewater – treatment – fatty acids

Ուսումնասիրվել է գարեջրի արտադրության թափոնների մաքրման հնարավորությունը *Parachlorella kessleri* և *Chlorella vulgaris* միկրոջրիմուռների շտամների օգնությամբ, ինչպես նաև հոսքաջրերի մաքրման արդյունավետությունը՝ կախված ինոկուլատի տարբեր ծավալներից: Հետազոտվել են նաև գարեջրի արտադրության թափոնների կիրառման հեռանկարները՝ որպես ճարպաթթուների արտադրության սուբստրատներ:

Միկրոջրիմուռներ – գարեջրի արտադրության թափոններ – վերամշակում – ճարպաթթուներ

Изучена возможность очистки сточных вод пивоваренных заводов штаммами микроводорослей *Parachlorella kessleri* и *Chlorella vulgaris*, а также эффективность очистки сточных вод в зависимости от различных концентраций объема инокулята. Также исследована возможность использования сточных вод пивоваренного производства в качестве субстрата для производства жирных кислот в рамках данного исследования.

Микроводоросли – сточные воды пивоваренного производства – очистка – жирные кислоты

Microalgae seem to be an alternative to the traditional biodiesel feedstocks such as edible vegetable oils or animal fats and other residual products from the economic, social and environmental point of view. Their high potential of biomass and fatty acids production and the possibility of using nutrients from waste streams (wastewaters and/or CO_2 flue gas emissions) can help reduce the environmental impacts and costs of cultivation. Moreover, microalgae are a feedstock for production of feeds, fertilizers, other fuels and chemicals.

Microalgae have an important role in the processes of industrial wastewater treatment. They are increasing the efficiency of removing biogenic elements, heavy metals, as well as eliminating pathogenic microflora. Microalgae can also utilize carbon dioxide released during the respiration of bacteria and produce oxygen necessary for heterotrophic aerobic bacteria to mineralize organic compounds.

The most important advantage of photosynthetic aeration is the cost reduction of the treatment facilities operation and the reduction of the high risk of volatile pollutants leakage that are associated with artificial aeration. Brewing is mostly a batch process. It is characterized by the so-called "burst emissions", when the concentration and amount of wastewater are liable to significant hesitations over time. The pH values of wastewater also fluctuate quite significantly, but usually remain neutral. During burst emissions wastewater is being enriched with used detergents and disinfectants, because of which the pH value can exceed 9.

Brewery wastewater usually has the following composition: residues of the final product, grains of barley, sprouts of malt, and particles of hop. The most polluted wastewater comes from grain soaking, hop extraction and yeast washing. They make up 27 % volume of all brewery wastewater. The yeast secessions of brewing give effluents with the following indexes: suspended solids - 500-2000 mg/L; BOD_5 (biological oxygen demand) - 1200-3000 mg O_2/L; nitrogen - 60-254 mg/L; phosphorus - 100 mg/L; potassium - 480 mg/L; pH 4-7.2.

Nitrogen in wastewater mostly comes from organic proteins and yeast income to the wastewater, and a small part of nitrogen comes from ammonia and nitrates. The quantitative content of nitrogen and phosphorus allows doing effective biological treatment of wastewater without the special addition of supplementary chemical reagents. The BOD_5 / nitrogen ratio in the total brewery effluent is 60-100, and BOD_5/COD (chemical oxygen demand) is approximately 0.6-0.7. These indicators are conducive for biological treatment processes of wastewater, which are based on nutrients removal mechanisms.

Microalgae have been used for wastewater treatment for several decades. At the same time, in recent years, a new direction has been developed for the organic and non-organic substances removing from wastewater by their accumulation in algal biomass, which is considered as an economically effective method of wastewater treatment [12; 13]. Microalgae are single-celled organisms that can be found in various aquatic environments. They are responsible for more than 40 % of the global carbon fixation, and are becoming more interesting nowadays because of their potential in biotechnology [1]. The growing interest is due to the fact that all microalgae have the capacity to produce energy rich oils especially in media with limiting substrates. These oils can then be used in production of renewable fuels, namely biodiesel, which can be obtained in a process called transesterification. Besides fatty acids, they also produce different products such are antioxidants, carotenoid, different polymers and peptides, which are used in different industrial products [14]. Microalgae can be cultivated in bioreactors and in open cultivation systems and there are several factors affecting their growth; light intensity, carbon source, temperature and different microelements and these are just a few of them. Among the aforementioned, temperature is one of the most important factors affecting the growth and which is very hard to control, especially in large–scale outdoor systems. The temperature oscillations in open pond or other types of cultivation systems can greatly affect lipid production. Certainly, different microalgae have different optimal temperatures for the growth but most of the species are able to withstand temperatures between 5-35^{0}C. One possible way to overcome this problem is to have closed control systems, photobioreactors, which can be used to increase bioprocess

yields and efficiency [8]. Due to their capability to accumulate lipids, microalgae are becoming popular in developing solutions for the replacement of fossil fuels which are becoming scarce. So far it has been shown that different substrates can be used for their cultivation especially wastewaters from different industries [5, 16, 18]. Some research has been done with the consortium of microalgae and bacteria [11] with promising results. Application of microalgae is slowly increasing so that some strains found their application even in human medicine, namely photodynamic therapy in treating tumor patients [9].

Chlorella and *Parachlorella* species are unicellular immotile green microalgae classified in the *Trebouxiophyceae*, which have spherical cells less than ~10 μm in diameter containing a chloroplast. Recently, these species have been used in many investigations where they showed good results for their lipid production. It has been shown that *P. kessleri* is able to accumulate lipids when sulphur source is a limiting component in cultivation media [17]. Besides, limitations in starch as carbon source, show similar results in experiments with this strain [6]. Another interesting fact about *P. kessleri* is that this microalga is also capable of producing H_2. Namely, by regulating light intensities and carbon source one strain was able to produce this alternative biofuel [7]. *Ch. vulgaris* on the other hand is a green eukaryotic microalgae in the genus *Chlorella*. It was discovered in 1890 by Martinus Willem Beijerinck as the first microalgae with a well-defined nucleus. As it contains a lot of protein, these microalgae have also found their application in human nutrition [2]. Japanese people are being recognized as the biggest consumers of *Chlorella*, especially because of its medical and health-improving properties. The protein content of *Ch. vulgaris* varies from 42 to 58% of its biomass dry weight [3] and they are considered to have a good nutritional quality, because algae are capable of synthesizing both essential and non-essential amino-acids [15]. Recently, these microalgae have also become interesting in the field of biofuels production. [10].

The aim of this study was to evaluate the prospects of primary treatment of brewery wastewater using different types of green unicellular microalgae, as well as the usage of wastewater as a nutrient medium for microalgae growth.

Materials and methods. ***Microalgae strains***

Two types of microalgae cultures: *Parachlorella kessleri* and *Chlorella vulgaris* (Culture collection of the Laboratory of Alternative Energy Sources of the Scientific and Production Center "Armbiotechnology" NAS RA) have been used within this research.

Nutrient media

The following nutrient media have been used during this experiment:

1. synthetic, chemically defined Tamiya's nutrient medium, with the following composition (g/L): KNO_3 - 2.0; KH_2PO_4 - 0.3; $MgSO_4 \cdot 7H_2O$- 0.3; trace element solution - 1ml; $Ca(NO_3)_2$ solution (14,4g/L) – 1ml. <u>*Trace element solution*</u> (g/L): $FeSO_4 \cdot 7H_2O$ - 5.0; $Co(NO_3)_2 \cdot 6H_2O$ - 0.02; $CuSO_4 \cdot 5H_2O$ - 0.01; $ZnSO_4 \cdot 7H_2O$ -0.04; $MnSO_4 \cdot H_2O$-1.12; H_3BO_3 - 0.6; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ - 1.062; Na EDTA -7.5. pH was adjusted to 9 using NaOH solution. Autoclaving: 1 atm., 121^0C, 20 min;
2. chemically undefined complex media: sterilized and non-sterilized brewery wastewater from "BEER OF YEREVAN" CJSC (Yerevan, Armenia).

Microalgae cultivation

40 Ml of previously cultivated microalgae (*Parchlorella kessleri* and *Chlorella vulgaris*) was added to 400 ml of nutrient media. Bubbling of cultural fluids was performed by aeration kits.

For the second part of experiment 10%, 30% and 50% v/v of inoculum was added to 200 ml of unsterilized wastewater. Flasks were cultivated on a rotary shaker at 28^0C and 150 rpm, exposed to artificial light source, respectively. Flasks were kept under the constant illumination (1500-1800 lux) at room temperature.

Determination of microalgae growth on the basis of spectrophotometric measurements

Growth rate was evaluated according to the samples optical density by spectrophotometric measurments at the wavelength of 540 nm. The samples for analysis have been taken every second day.

Dry biomass obtaining

After two weeks of cultivation, the algal cells were harvested using centrifuge (6000 rpm, 10 min). Then, the wet biomass was dried in thermostat (40^0C) until constant weight was obtained.

Lipid extraction

The extraction of lipids was performed by Bligh and Dyer method [4] using 50 mg sample.

Determination of fatty acid content

The composition of fatty acids was determined by gas chromatography. Prior to GC analysis, the samples were re-suspended with 1 ml of hexane and transferred into 3 ml Eppendorf cuvettes. For this analysis we had to obtain fatty acid esters by adding 1 ml of methanol.1μL of sample was used for the analysis of fatty acid composition. The fatty acid methyl esters have been determined by the Shimadzu GCh-2010 gas chromatography saturated with DB-5-MS capillary column in the 80-2904^0C temperature range. The temperature of the column was increased 40^0C/min. The temperatures of injector and detector were 250 and 230^0C, respectively. Carrier gas flow was 1.2ml/min.

Amino-acid composition analysis

The amino acid range of brewery wastewater was determined by amino acid analyzer "Shimadzu Nexera X2" (Japan). For separation of amino acids Novo-Pak C 18.4 μm, 3.9-150 mm chromatographic column was used. It was carried out in a gradient elution mode. The following reagents were used as the mobile phase: A). acetonitrile: methanol: water (45:40:15 -v/v); B). phosphate buffer pH = 7,0; flow rate was 0.5 ml / min, detection was carried out at a wavelength of ex350-em450 nm, column temperature – 300^0C, injection volume - 10 μl; chemical reagents and eluents: MeCN, MeOH, Na_2HPO_4, NaH_2PO_4, HCI, ortho-phthaldialdehyde reagent CAS: 643-79-8 (Sigma-Aldrich with purity> 99.9 %).

Determination of the total content of organic and non-organic matter in brewery wastewater

The total content of organic and non-organic substances in the wastewater was determined by the following method: 5 ml of wastewater samples were placed in pre-weighed crucibles. The crucibles with the samples were put in a thermostat (80^0C). After 24 hours the crucibles were weighed, the operation was repeated until obtaining the constant weight. To determine the total inorganic content, the sample was placed in a muffle oven (550^0C, 1 hour), and the experiment was repeated until the constant weight was obtained.

Results and Discussion. The analysis of the total content of organic and non-organic matter in brewery wastewater showed that the dry matter content (organic + non-organic) in the studied wastewater sample was 4.4±0.24 mg/ml and the ash content (non-organic matter) was 1.12±0.0 mg/ml. That is, the content of organic matter was 3.28 mg /ml. Prior to cultivation, amino acid analysis of wastewater has been done. The results showed that only 3 amino acids were present in brewery wastewater (fig. 2).

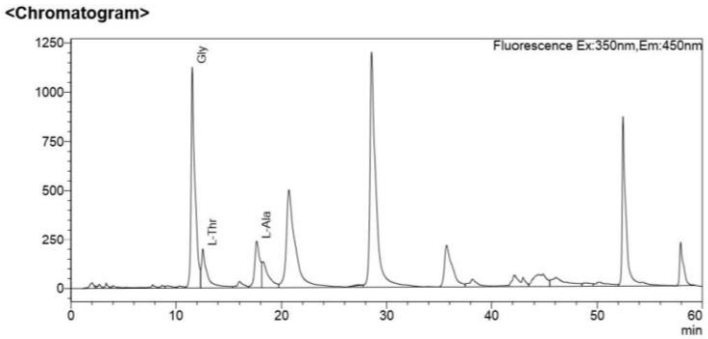

Fig. 1. Chromatogram of amino acid analysis of wastewater The calculations showed the following content of determined amino acids (μg/ml): Glycin-4; L- Threonine-3; L- Alanine-6.

The microalgae growth assessment has been done for 14 days, and then the cultivation was stopped. The results of spectrophotometric measurements were summarized and the kinetic curves were established (fig. 2 and 3).

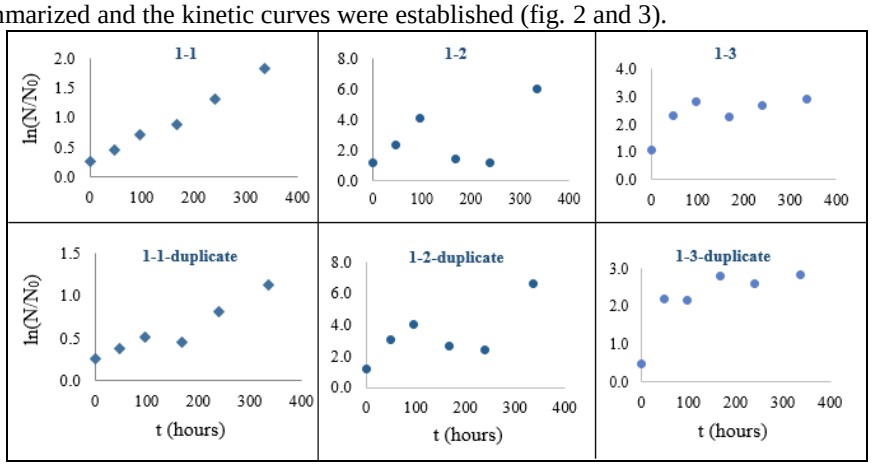

Fig. 2. Growth curves of P. kessleri in
1-1.Tamiya's nutrient medium, 1-2. Sterilized wastewater, 1-3. Non-sterilized wastewater

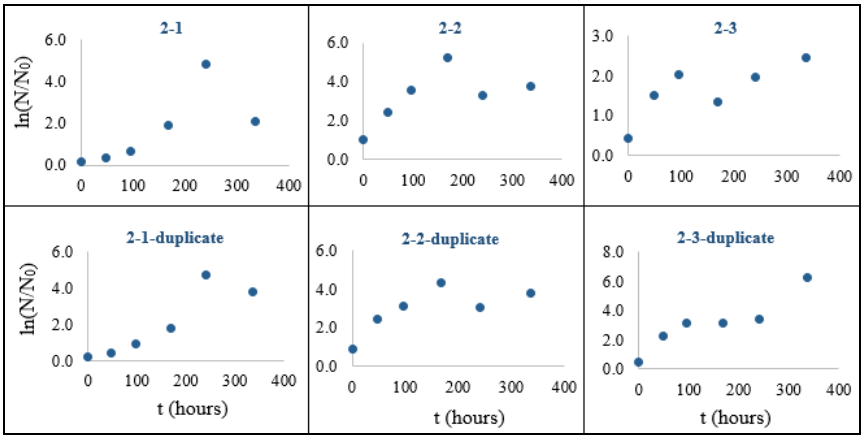

Fig.3. Growth curves of Ch. vulgaris in Tamiya;s nutrient medium
2-1. Tamiya's nutrient medium; 2-2. Sterilized wastewater; 2-3. Non-sterilized wastewater

According to the presented data the microalgae growth was observed in all samples. However, the results do not still allow to draw any definite conclusions, especially about the growth of microalgae in non-sterile wastewater samples. The accompanying microflora in the wastewater may interfere with obtaining correct results and the optical density changes can be caused by the growth of other microbes, present in wastewater, including the growth of residual yeast. However, the experimental results suggest that *P. kessleri* microalga grows better on wastewater, which may be due to several reasons: higher rate of this alga growth, when the bacterial growth was simply suppressed, and the chemical composition of wastewater, which was more optimal for this species. The analysis of the results showed that sterilized wastewater was a better medium for the growth of *Ch. vulgaris*, while *P. kessleri* microalga showed similar growth in both sterile and non-sterile ones. This means that the use of *P. kessleri* for wastewater treatment may be more promising, since the usage of non-sterile effluents for

the cultivation of microalgae can significantly reduce the cost of bioprocess, especially on a large-scale, which is a valuable research result.

The importance of the inoculum volume on the growth rate of microalgae was studied in the next step of the research. 10; 30 and 50 % v/v of inoculum volumes were taken and added to 200 ml of non-sterilized brewery wastewater. The microalgae growth rate was monitored within 16 days. The results of the spectrophotometric measurements are summarized in figures 4 and 5.

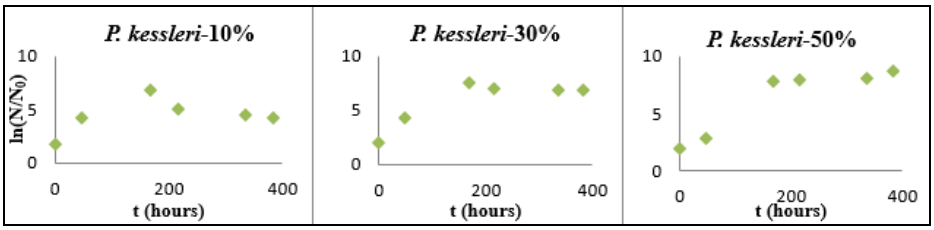

Fig.4. Effect of different inoculum volumes on *P. kessleri* growth in non-sterilized wastewater

According to the data obtained, the increasing of inoculum volume leads to suppression of the growth of external microflora and, as a consequence, to an increase in the growth rate of microalgae.

The analysis of the results showed that for *P. kessleri* the best results were obtained in the experiment with 50 % v/v of added inoculum. In the case of adding 30 % v/v of inoculum quite good results were also observed. In the stationary phase of the growth of *P. kessleri* showed almost the same growth rate as with 50 % inoculum.

Similar results were obtained in the experiments with *Ch. vulgaris* (fig. 5), where once again the experimental data show that the larger the volume of inoculate, the more efficient the growth of microalgae, and consequently, the treatment of wastewater.

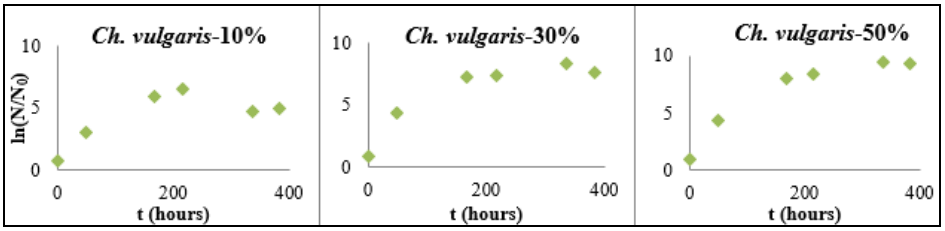

Fig.5. Effect of different inoculum volumes on Ch. vulgaris growth in non-sterilized wastewater

The main goal of this investigation was to determine the potential of brewery wastewater as a substrate for algal biomass production, and therefore the potential for algal fatty acid production. Based on our results, the best media for algal growth was sterilized synthetic Tamiya's nutrient medium, so it was expected to have the highest fatty acid content in this medium. In this chemically defined nutrient medium different types of fatty acids were detected using gas chromatography: lauric, myristic, palmitic, stearic, oleic and linoleic fatty acids. The most abundant fatty acids were palmitic acid (25 %) and linoleic acid (18 %).

In the case of *P. kessleri* cultivation in both sterilized and non-sterilized wastewater, the total amount of fatty acids was significantly lower which was in correlation with spectrophotometric measurements. However, palmitic acid in these

samples was also the most abundant (50 %). In the case of sterilized wastewater oleic acid (10 %), linoleic acid (30,9 %) and stearic fatty acid (9,1 %) were also determined.

The analysis of these results once again shows that the obtained high OD values are probably the result of growth of accompanying microflora.

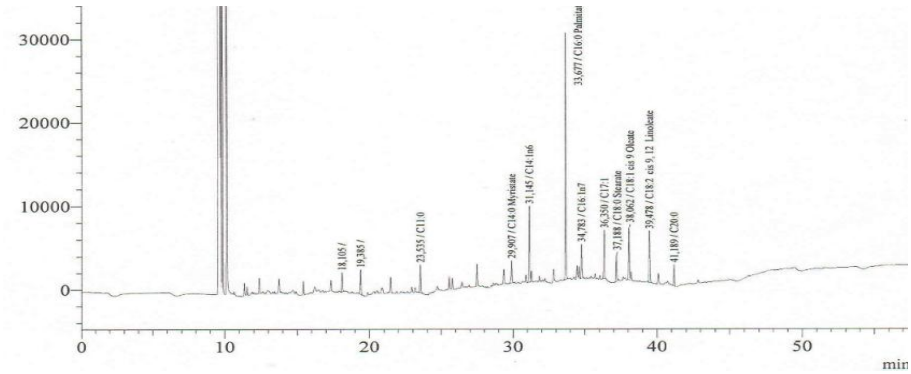

Fig.6. Chromatogramm of *P. kessleri* microalgae biomass fatty acid composition (non-sterilized wastewater, 50% inoculum)

The total amount of produced fatty acids was less in the case of *Chlorella vulgaris* compared to *P. kessleri*. The highest concentrations of fatty acids found in cultivated in Tamiya's nutrient medium samples. There were palmitic, oleic and linoleic acids, with 58.8 %; 14.7 % and 26.5 % concentrations, respectively. Similar results concerning the composition of fatty acids were also obtained in the case of cultivation in sterilized brewery wastewater. The only difference was the detected concentration of stearic fatty acid with 10.5 % in total fatty acid content. The obtained results are also in a correlation with spectrophotometric data. The lowest amount of produced fatty acids was observed in the experiment with non-sterilized wastewater: only two fatty acids were detected with very low concentrations.

Regarding the studying of the fatty acid composition of biomass obtained in different volumes of inoculum, it turned out that in the case of 30% inoculum *Ch. vulgaris* was a better fatty acid producer than *P. kessleri* (palmitic acid: 77.5 %, oleic acid: 9.1 %, stearic acid: 8.3 % and linoleic acid: 5,1 %). At the same time in the case of 50 % of inoculum *P. kessleri* was a better fatty acid producer than *Ch. vulgaris* (the contents of myristinic and stearic acids were almost equal, linoleic acid was found only in biomass of *P. kessleri*, and in both samples palmitic acid was predominant).

Based on the results of this research, it can be concluded that both: the chemically defined nutrient medium and brewery wastewater are good media for microalgae growth, and therefore are valuable substrates for the production of fatty acids. The obtained results of research have shown that even non-sterilized media can be used for the cultivation of microalgae but a larger volume of inoculum should be used. These results suggest that sterilization step may be skipped and therefore it could reduce the energy requirement of the bioprocess.

Acknowledgment

This work was carried out within the frames of Grant Project 19YR-1F051 (Science Committee of the RA MoESCS).

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Received on 02.11.2020