

THE ETHANOL INDUSTRY WASTE AS A VALUABLE FEEDSTOCK
FOR HYDROGEN PHOTOPRODUCTION BY GREEN ALGAE
CHLORELLA VULGARIS

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Green algae are promising objects of biotechnology due to their high growth rates and the ability to produce biologically active compounds. Moreover, green algae are able of generating biohydrogen (H₂), which is of interest in biofuels technology. In this study an application of distillers grains with solubles (DGS), the main waste generated during ethanol production, as a feedstock for enhancing the yield of H₂ in green alga *Chlorella vulgaris* IBCE C-19 was investigated. The results obtained showed the possibility of using of this waste as a valuable source of natural biological compounds in H₂ production.

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Keywords: green algae, ethanol industry waste, biohydrogen, redox potential.

Introduction. Biohydrogen (H₂) production from various substrates is considered as one of the promising ways to generate ecologically clean and renewable energy and can have a crucial role in alternative energy technologies [1, 2]. Nowadays, one of the important aspects of hydrogen energetics is the selection of microorganisms, which are able to produce H₂ effectively and the optimization of conditions to provide high yield of H₂. H₂ production by green algae has a great interest due to various advantages such as natural origin, efficiency and renewability of solar energy and the substrate – water, as well as having non-toxic by-product – oxygen [2–4]. Green algae generate H₂ during photosynthesis using sunlight as an energy source [4, 5]. H₂ evolution by photosynthetic microorganisms is enhanced by addition of external organic carbon sources [6]. The selection of the carbon source for microorganism's cultivation is a serious problem, because it strongly affects the growth properties and biomass yield.

Nowadays, constantly increasing demand for energy needs new and cheaper sources. Various industrial wastes can be a new promising approach of H₂ production [7–11]. Distillers grains with solubles (DGS) are the main waste generated during ethanol production [12–14]. This waste contains various organic acids (succinate, acetate and lactate), sugars (xylose, glucose and arabinose), proteins, different amino acids including 8 essential amino acids (arginine, lysine, valine, histidine, threonine, phenylalanine, leucine, isoleucine), vitamins and trace elements (calcium, iron,

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magnesium, manganese, etc.) [12–14]. Thus, DGS can be used as valuable and attractive feedstock for obtaining of biomass and H₂ in microorganisms. Moreover, this waste is very cheap, its cost in Armenia is 1.5 cent per liter [11, 15]. In our previous studies it has been shown that purple bacteria *Rhodobacter sphaeroides*, isolated from Armenia, produce H₂ during photofermentation of various industrial wastes [10, 15].

Moreover, the application of ethanol industry waste in H₂ production by green algae has not been investigated up to date. In this case the use of green algae and DGS is important as a new approach for the development of H₂ biotechnology. However, the mechanisms and optimal conditions benefiting H₂ photoproduction by algae from ethanol industry waste should be also analyzed.

During this work the H₂ generation ability in *Chlorella vulgaris* IBCE C-19 using ethanol production wastes, DGS as valuable feedstock was investigated for the first time. Selection of optimal conditions (pH and dilution of wastes) increasing biomass and H₂ yield in algae is performed. In order to investigate the mechanisms of H₂ photoproduction in the mentioned conditions, the effects of PS II inhibitor diuron on H₂ yield in green algae is also determined.

Materials and Methods.

Algae Cultivation Conditions and Determination of Growth Properties.

Green algae *C. vulgaris* IBCE C-19 (Algae Collection, Institute of Biophysics and Cellular Engineering of the National Academy of Sciences, Minsk, Belarus) was cultivated under aerobic conditions (Fig. 1, A) in Tamiya medium with acetate as a carbon source and upon continuous illumination ($\sim 50 \text{ W}\cdot\text{m}^{-2}$) [16, 17]. The algae growth was determined by Spectro UV-Vis spectrophotometer (Labomed, USA) by measuring the optical density (OD₆₈₀) [17]. The growth rate was calculated as described [17]. The pH of growth media was determined by a pH-meter (“HANNA Instruments”, Portugal) applying appropriate electrodes [10, 16].

Photosynthetic pigments (chlorophylls (Chl) *a* and *b*, and carotenoids) were extracted from the algae cells using 96% ethanol; pigments content in the extracts was calculated according to [16, 17]. Absorption spectra of algal cell extracts were measured in the 400–800 nm wavelength regions using Spectro UV-Vis spectrophotometer (“Labomed”, USA) interfaced to a personal computer [16].

The Redox Potential and H₂ Yield Assay, Pretreatment of Ethanol Industry Waste. The redox potential of algae growth medium was measured during anaerobic growth by using a pair of redox (platinum and titanium-silicate) electrodes (“Measuring Instruments Enterprise”, Gomel, Belarus) as described [10, 16]. The H₂ yield in *C. vulgaris* was defined as described in [16, 17]. For H₂ production assay *C. vulgaris* cells, grown aerobically, were harvested in the late logarithmic growth phase (10^6 cells/mL) by centrifugation (2000 rpm, 10 min), washed twice, and then resuspended in Tamiya media (pH 7.5) under anaerobic conditions [16]. The algae culture grown in Tamiya medium was used as a control.

For study the H₂ generation by algae using ethanol industry waste, DGS, the algae cells were moved after centrifugation in waste containing media under anaerobic conditions (Fig. 1, B). DGS was obtained from “Alex Grig” Alcohol Plant Co. LTD (Yerevan, Armenia). The untreated waste was filtered through cotton wool and paper filter, next sterilized by autoclaving [15]. Before autoclaving the pH of the

DGS was adjusted to 7.5, and DGS were diluted 2, 5 and 10-fold using distilled water (Fig. 1, C).

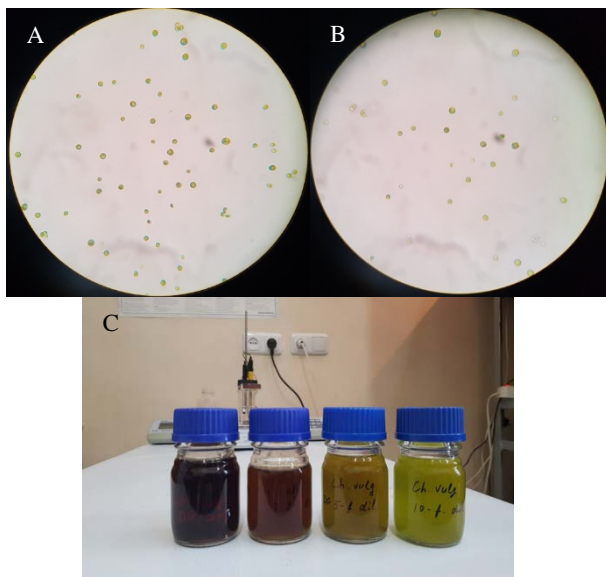


Fig. 1. Light microscopy image of *C. vulgaris* C-19 ($\times 1000$, Microscope XSP-136C, China), cultivated under aerobic conditions in Tamiya medium (A) and anaerobic conditions in 5-fold diluted DGS medium (B); microalgae culture, grown in undiluted DGS and DGS media with various dilutions (C).

To determine the PS II inhibitor effect, diuron ($30 \mu\text{M}$) was added to the algae media after 24 h of cultivation under anaerobic conditions [16]. Then the cells were maintained in the dark for about 15 min, after which they were moved under illumination.

Reagents and Data Processing. Different reagents (“Carl Roth GmbH”, Germany; “Sigma Aldrich”, USA; “UNI-Chem”, China) of analytical grade were used. Standard deviations were calculated using appropriate function of Microsoft Excel 2016 [10, 17]. The changes were validated by calculating the Student’s validity criteria ($p < 0.05$).

Results and Discussion.

Determination of *Chlorella Vulgaris* Growth Properties During Growth in DGS Media. Algae of genus *Chlorella* are promising objects for biotechnology and can be used in different industrial fields. Suspension of *Chlorella* contains up to 60% proteins, 30% carbohydrates, 5–10% lipids, and up to 10% of various microelements: iron, calcium, phosphorus, zinc, manganese, etc. [4, 18]. Green algae are also able to generate hydrogen, which is associated with electron transfer during photosynthesis and catalyzed by hydrogenase [5, 16].

In this work the H_2 generation ability in *C. vulgaris* using ethanol production wastes, DGS, as valuable feedstock was investigated for the first time. *C. vulgaris* is cultivated in Tamiya medium under aerobic conditions upon illumination in the presence of acetate as a carbon source (Fig. 1, A). Then cells of algae were moved in Tamiya media and 2, 5 and 10 fold diluted DGS media (Figs. 1, B and C), where the anaerobic conditions were maintained, because H_2 generation in this alga is not observed under aerobic conditions.

The results showed that H_2 generation in green algae, grown in DGS media was started at 24 h growth and continued up to 96 h growth, whereas in control cell,

cultivated in Tamiya medium, H₂ production was detected at 48 h. Moreover, no H₂ generation was observed, when undiluted DGS were used. It is coupled with high organic compounds content of this waste [12–14]. In this case, dilution of DGS is required to optimize the DGS compounds concentration. Moreover, neutralization of the pH of DGS is also necessary, since the pH of DGS was ~4.0, the pH of the waste was adjusted to 7.5 before the cultivation of the algae.

The results obtained showed that during the algae growth in 2, 5 and 10 fold diluted DGS media H₂ the production by *C. vulgaris* was higher in comparison with the control (Fig. 2, A). The highest H₂ yield was detected during 48 h growth in 5 and 10 fold diluted DGS, which was ~3 fold higher than H₂ yield in the control, grown on Tamiya medium (Fig. 2, A).

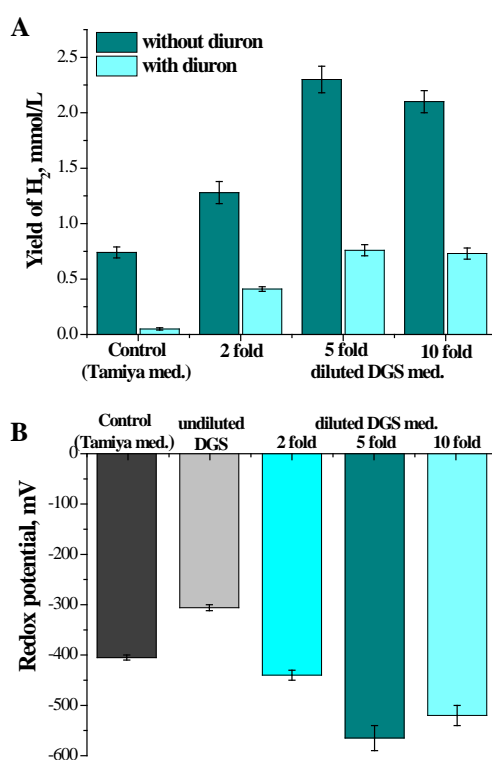


Fig. 2. The H₂ yield (A) and redox potential (B) in *C. vulgaris* IBCE C-19 cultivated in Tamiya (control) and 2–10 fold diluted DGS media.

To determine the contribution of PS II in H₂ generation process during algae growth in waste containing media the effect of diuron, a known inhibitor of photosystem (PS) II activity was investigated. Diuron blocks the transfer of electrons from PS II reaction center to Cyt b₆f complexes and inhibits the ATP synthesis [16, 17]. Diuron inhibited H₂ generation process in control cells grown in Tamiya medium. However, diuron suppressed the H₂ generation in *C. vulgaris* by ~70% in

5 and 10 fold diluted DGS (Fig. 2, A), which indicates the significant role of PS II as a supplier of electrons (~70%) for H₂ production in this algae, this pathway of H₂ generation is known as PS II-dependent pathway [5, 16]. The effect of diuron on H₂ yield in diluted DGS media indicates that the main electrons for H₂ generation are provided by PS II during splitting of water, however ~30% of electrons to hydrogenase are supplied by PS II-independent way. Thus, DGS as a mixture of organic acids and sugars is a preferable exogenous donor of electrons than acetate, which is used as a carbon source in Tamiya medium.

H₂ production by microorganisms is coupled with decrease in the value of the redox potential value [9, 10, 16]. The redox potential of *C. vulgaris* control cells, grown in Tamiya medium, decreased during 72 h up to $(-405 \pm 5 \text{ mV})$ (Fig. 2, B). During cultivation of algae in undiluted DGS the decrease in the redox potential was observed (Fig. 2, B) to $(-306 \pm 5 \text{ mV})$, whereas in diluted DGS media, the drop in the redox potential was more pronounced (Fig. 2, B). In 5 and 10 fold diluted DGS media the redox potential decreased up to $(-565 \pm 25 \text{ mV})$ and $(-520 \pm 20 \text{ mV})$, respectively (Fig. 2, B). Diuron delayed drop in redox potential (not shown).

It is known, that the H₂ production by green algae is observed during the photosynthesis [4, 5, 16]. To determine the photosynthetic pigments formation during growth in diluted DGS media, absorption spectra of *C. vulgaris* cell extracts in ethanol were obtained (Fig. 3). The photosynthetic pigments of green algae include Chl *a* and *b*, as well as carotenoids, which participate in the absorption of light energy [16]. The measured absorption spectra (Fig. 3) show that the level of total carotenoids and chlorophylls in *C. vulgaris* grown in a 5 fold diluted DGS was reduced in comparison with the control. A decrease in the content of Chl *a* by ~35 and 53% was detected in 5 and 10 fold diluted DGS media, respectively, compared with the control (Fig. 4). Chl *b* content decreased by ~40 and 47% in 5 and 10 fold diluted DGS media, respectively (Fig. 4). The total carotenoids concentration decreased up to ~57 and 65% during cultivation of *C. vulgaris* in 5 and 10 fold diluted DGS media, respectively (Fig. 4). The results indicated that the cultivation in diluted DGS leads to a decrease in the content of photosynthetic pigments.

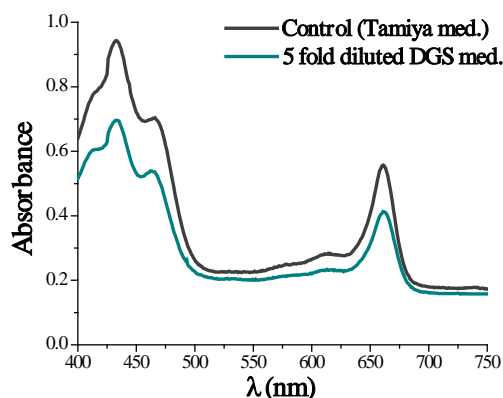


Fig. 3. The absorption spectra of ethanol extracts of *C. vulgaris* IBCE C-19 cell cultivated in Tamiya (control) and 5-fold diluted DGS media.

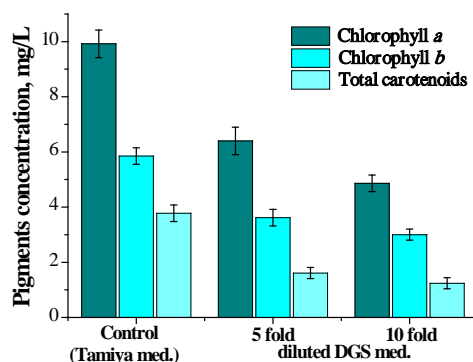


Fig. 4. Changes of photosynthetic pigments content in *C. vulgaris* IBCE C-19 cultivated in Tamiya (control) and diluted DGS media.

Conclusion. In the current study the prospects of application of ethanol production wastes to obtain hydrogen by *C. vulgaris* was investigated for the first time. The results obtained showed that *C. vulgaris* can use DGS as a valuable feedstock for hydrogen generation, moreover, the H_2 photoproduction in DGS media was 3 fold higher than in the control media. Thus, ethanol industry waste requires pretreatment for effective utilization by green algae, including neutralization (pH 7.5) and appropriate dilutions. Therefore, the use of DGS in biohydrogen technology provides not only a valuable and cheap source of H_2 , but also helps to solve the problem of waste utilization.

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Ջ. Գ. ՄԱՆՈՅԱՆ, Լ. Ս. ԳԱԲՐԻԵԼՅԱՆ

ԷԹԱՆՈԼԻ ԱՐՏԱԴՐՈՒԹՅԱՆ ԹԱՓՈՆԸ՝ ՈՐՊԵՍ ԿԱՆԱԶ ՋՐԻՄՈՒՌ
CHLORELLA VULGARIS-ՈՒՄ ՋՐԱԾՆԻ ՖՈՏՈԱՐՏԱԴՐՈՒԹՅԱՆ
ԱՐԺԵՔԱՎՈՐ ՀՈՒՄՔ

Կանաչ ջրիմուռներն իրենց անման բարձր արագության և կենսաբանորեն ակտիվ միացություններ արտադրելու ունակության շնորհիվ կենսատեխնոլոգիայի հեռանկարային օբյեկտներ են: Ավելին, ջրիմուռները կարող են արտադրել կենսաջրածին (H_2), որը հետաքրքրություն է ներկայացնում կենսավառելիքի արտադրության տեխնոլոգիայում: Տվյալ աշխատանքի ընթացքում հետազոտվել է էթանոլի արտադրության թափոնի որպես հումքի կիրառությունը՝ կանաչ ջրիմուռ *Chlorella vulgaris* IBCE C-19-ում H_2 -ի էլքի խթանման համար: Ստացված տվյալները ցույց են տվել կենսաջրածնի արտադրության համար էթանոլի թափոնի՝ որպես բնական կենսաբանական միացությունների արժեքավոր աղբյուրի, օգտագործման հնարավորությունը:

Д. Г. МАНОЯН, Л. С. ГАБРИЕЛЯН

ОТХОД ПРОИЗВОДСТВА ЭТАНОЛА КАК ЦЕННОЕ СЫРЬЕ ДЛЯ
ФОТОВЫДЕЛЕНИЯ ВОДОРОДА ЗЕЛЕННОЙ ВОДОРОСЛЬЮ
CHLORELLA VULGARIS

Зеленые водоросли благодаря высокой скорости роста и способности продуцировать биологически активные соединения являются перспективным объектом биотехнологии. Более того, водоросли способны генерировать биоводород (H_2), что представляет интерес для технологии производства биотоплива. В ходе данной работы было исследовано применение барды – основного отхода, образующегося при производстве этанола, в качестве сырья для увеличения выхода H_2 зеленой водоросли *Chlorella vulgaris* IBCE C-19. Полученные результаты показали возможность использования барды в качестве ценного источника природных биологических соединений для производства H_2 .