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# VALORIZATION OF LIGNOCELLULOSIC BIOMASS BY DARK FERMENTATION: DEVELOPMENT OF EFFECTIVE CONDITIONS FOR MOLECULAR HYDROGEN BIOSYNTHESIS

***Abstract:*** *Valorization of lignocellulosic biomass (LB) is important to reduce their environmental impact and reduce the risk to human health. Conventional methods for handling secondary raw materials primarily focus on waste disposal, treating lignocellulosic biomass as waste rather than as a source of organic substances for producing value-added products. As an alternative, processes should be developed to add value to waste, producing value-added products with economic and environmental benefits. In this regard, studies have focused on operating parameters, pretreatment, and microbial fermentation to enhance hydrogen yield during dark fermentation. Upper (4%) and lower (20%) concentrations of distillery grain based substrates for biohydrogen synthesis using wild-type E. coli have been established. Conditions for rational formation of reducing sugars by varying feedstock and acid concentrations in distillery grain based substrates have been established. During the study of the effect of acid-hydrothermal treatment of stillage on the total yield of biohydrogen, it was found that the optimal concentration of sulfuric acid is 1,5%, while 10% of the raw material is used. Under these conditions, the maximum yield of molecular hydrogen was achieved, equal to 116 ±1,0 ml/l using wild-type E. coli.*

***Key words:*** *Valorization, distiller's grains, pre-treatment, dark fermentation, sugars, molecular hydrogen.*

**Introduction**

Lignocellulosic biomass is a common and cost-effective feedstock. It mainly consists of cellulose (33-55 wt %), hemicellulose (20-40 wt %), and lignin (10-25 wt %) [1]. Using lignocellulosic components as carbon and nutrient sources for the growth medium can reduce the cost of producing value-added products, increasing their competitiveness. Dark fermentation is widely recognized as an effective method for producing biohydrogen using biological processes [2]. Hydrogen is an attractive energy carrier due to its high energy density and is used as a feedstock in various chemical and food processes. In this regard, biohydrogen production has been actively researched by advanced research centers around the world in the past decade.

A thorough and comprehensive understanding of dark fermentation of lignocellulosic waste not only contributes to sustainable development and circular economy, but also opens up new opportunities for more efficient and environmentally friendly processes. The main components of polysaccharides, which make up a significant part of lignocellulosic biomass, are glucose and xylose. The conversion of xylose and glucose during the pretreatment step results in the formation of various compounds, including furfural and 5-hydroxymethylfurfural (HMF). Furfural and HMF are well known for their inhibitory effects in the field of microbial conversion [3]. Therefore, they hinder the final biohydrogen production process. In addition, pretreatment destroys hydrogenotrophic methanogenic archaea, which are H2 consumers, in order to subsequently enrich the community with hydrogen-producing organisms [4].

During dark fermentation, biohydrogen is formed by two mechanisms. One of them occurs due to the catabolic transformation of formic acid, and the other is due to the re-oxidation of nicotinamide adenine dinucleotide hydride (NADH), which is catalyzed by the hydrogenase pathway [5]. Theoretically, the complete conversion of 1 mole of glucose leads to the formation of 12 moles of hydrogen atoms.

$$C_6H_{12}O_6 + 6H_2O = 12H_2 + 6CO_2 \quad (1)$$

However, it has been reported that the practical yield of hydrogen during dark fermentation only reaches up to 4 mol [6]. In this case, the by-products of the reaction are organic acids [7]:

$$C_6H_{12}O_6 + 6H_2O = 2CH_3COOH + 4H_2 + 2CO_2 \quad (2)$$

Accordingly, this technology demonstrates the efficiency and ability to meet future needs for valorization of lignocellulosic biomass by producing various value-added products. This is also evidenced by the fact that biohydrogen produced by dark fermentation has a comparatively higher rate and quantity than other methods. [8, 9].

In accordance with the UN strategy for sustainable development, one of the most serious challenges related to environmental, economic and social aspects is the minimization and valorization of agricultural processing waste [10].

According to statistics, there are 142 producers of ethyl alcohol and alcoholic beverages in Kazakhstan, seven of which produce only ethyl alcohol: LLP «Gold Pegasus» (Shymkent), LLP «Tau-Product» (Shymkent), LLP «ALPHA-2050» (Kostanay), LLP «Talgarspirt» (Talgar), LLP «entaur» (Kandyagash), LLP «Maximus» (Aktobe), JSC «Aidabulsky alcohol plant» (Aidabul v., Zerendi district) and LLP «Alfa Organic» (Stepnogorsk) [11]. Most of these plants currently operate on a “single-product” scheme, where only alcohol is obtained from the main raw material as a commercial product. At the same time, other components of the raw material go to waste. In Kazakhstan, with an average annual production of 18 million liters of ethyl alcohol, up to 2.43 million tons of distillery grain are released. However, it is likely that the numerical values for such an assumption vary depending on the production process used, although there is no special study indicating these ranges. It should also be noted that these by-products are perishable products, the shelf life of which is no more than 24 hours from the moment of production, after which the stillage sours, loses its useful properties and toxins accumulate in it.

The current industrialization model is insufficient to maintain a safe and clean environment. Therefore, there is a need to create waste valorization systems in industry [12]. In accordance with the principles of bioeconomics, stillage should be converted into value-added products, including a unique product – biohydrogen. It should be noted that a small part of domestic plants process post-alcohol stillage into dry feed or fertilizer. Direct use of stillage as a feed additive for farm animals is carried out only partially through its purchase in its native form by entrepreneurs working in rural areas or private owners. However, such use is not effective, since it requires additional processing and is seasonal, which creates problems with storage and transportation costs.

**Materials and methods**

The mass fraction of carbohydrates in the raw material was determined by the permanganate method, based on the ability of reducing sugars to reduce copper oxide to copper oxide in an alkaline solution. Sugars were determined by reducing iron (III) with copper (I) and subsequent titration of iron (II) with potassium permanganate [13]. The analysis of total dry matter (TS) and volatile solids (VS) was carried out in accordance with the work [14].

The carbohydrate composition of the distillery grain and its substrates was determined by high-performance liquid chromatography (HPLC) [15]. Organic acids in the distillery dregs and its substrates were determined by capillary electrophoresis [16]. The presence of furfural in the substrates was determined according to GOST 32013-2012. Furfural in the substrates was determined by the HDR method based on the reaction of furfural with hydroxylamine hydrochloride. Oxymethylfurfural in the substrates was determined according to GOST 19792-2001 [17].

To perform preliminary treatment and microbial fermentation, the following operations were performed:

- Preparation of the distillery grain solution by mixing it with distilled water and treating it with sulfuric acid with a fraction for 5±1 minutes;
- Carrying out acid-hydrothermal treatment using an autoclave at a temperature of 121 ° C (1.1 atm) and with a duration of 20 minutes;
- Filtration of solid residues from the hydrolyzate by separation using a set of sieves with hole diameters of 0,01÷1,0 mm;
- Neutralization of the hydrolyzate using KOH to pH 7,5;
- Mechanical purification of hydrolyzate from colloidal impurities by centrifugation at g=3782, w=4200 rpm; t=4°C; $\tau_{center.}$ =15 мин;
- Final processing of hydrolyzate using an autoclave at a temperature of 121°C (1,1 atm) and with a duration of 20 minutes;
- Preparation of inoculum (bacterial culture) with the composition: peptone – 20 g/l; potassium hydrogen phosphate – 2 g/l; sodium chloride – 5 g/l. The temperature of bacterial culture cultivation is t=37 °C for 18÷20 h.
- Introduction of inoculum into hydrolyzate prepared from stillage with a volume fraction of 3%;
- Synthesis and production of biohydrogen by anaerobic fermentation with *E. coli* strains at a temperature of 37 °C.

**Results and discussions**

As part of the study, an analysis of the biochemical composition of the distillery grains (Table 1) obtained from the «Alfa Organic» Malt Distillery LLC (Akmola Region, Stepnogorsk) was conducted.

Table 1 – Biochemical composition of distillery grains

| Name of indicators, units of measurement | Actual results | Regulatory documentation on test methods |
|---|---|---|
| Physicochemical parameters: | | |
| – moisture content, % | 90,57 | GOST 13496.3-92 |
| – carbohydrate content, % | 10,25 | Permanganate method |
| – protein content, % | 4,045 | GOST 13496.4-93 |
| – ash content, % | 0,21 | GOST 26226-95 |
| Carbohydrate composition, %: | | GOST 13496.3-92 |
| – maltose content | 3,82 | |
| – glucose content | 4,53 | |
| – fructose content | 4,09 | |
| Organic acids, mg/l | | M 04-47-2007 |
| - oxalic acid<br>- lactic acid<br>- succinic acid<br>- - acetic acid | 0,395<br>0,850<br>0,215<br>0,055 | |

Due to its high carbohydrate content, distillery grain is the most suitable raw material for the production of bioproducts using dark fermentation. The efficiency of dark fermentation of

carbohydrate-containing waste in the process of biohydrogen production depends on several key factors: the composition of the substrate, microorganisms, parameters and conditions of pre-treatment (temperature, pressure, duration of hydrolysis, pH,) and microbial synthesis, etc. The composition of carbohydrate substrates can be more or less suitable for bacteria involved in the dark fermentation process. Therefore, in order to achieve maximum efficiency of dark fermentation of carbohydrate waste, it is necessary to study the conditions for effective biohydrogen synthesis.

In this regard, the effect of acid-hydrothermal treatment of distillery grain on the efficiency of microbial fermentation was studied. The concentrations of raw materials and sulfuric acid, and the duration of acid hydrolysis were determined as variable factors of acid-hydrothermal treatment of distillery grain. In accordance with this, experiments were conducted to determine the smallest and largest values of the variable factors in the release of biohydrogen. In particular, the minimum and maximum values were established: the concentration of distillery grain (4÷20%) and sulfuric acid (0,75÷3%), the duration of acid hydrolysis (120÷240 minutes). The efficiency of biohydrogen synthesis was assessed by measuring the following parameters: (1) the oxidation-reduction potential of the substrate using an ion meter with a platinum electrode, which allows measuring the oxidation power of hydrogen ions; (2) the cumulative yield of biohydrogen.

The results of the study of dark fermentation of a substrate based on a 4% fraction of distillery grain using wild-type *E. coli* showed that when using sulfuric acid with a fraction of: (i) 0,75%, biohydrogen molecules are synthesized in the period from 3 (±0,05) to 6 (±0,05) hours of fermentation; (ii) 1,5%, biohydrogen molecules are synthesized only at 6 (±0,05) hours of fermentation; (iii) 3%, biohydrogen molecules are synthesized in the period from 6 (±0,05) to 24 (±0,05) hours of fermentation.

The results of the study of dark fermentation of a substrate based on a 20% fraction of distillery grain using wild-type *E. coli* showed that when using sulfuric acid with a fraction of: (i) 0,75%, biohydrogen molecules were not detected during the fermentation period; (ii) 1,5%, biohydrogen molecules were synthesized in the period from 3 to 6 hours of fermentation; (iii) 3%, biohydrogen molecules were synthesized only at 6 (±0.05) hours of fermentation.

Thus, the maximum and minimum values of the proportion of raw materials and acid in the substrate based on stillage for the synthesis of biohydrogen using wild-type *E. coli* were determined:

– In the case of using a substrate based on a 4% fraction of distillery grain, the longest synthesis (6-24 (±0,05) hours) of biohydrogen was achieved in a sample where the treatment was carried out with sulfuric acid with a fraction of 3%. In this case, the pH of this sample during the biohydrogen synthesis period changed from 5,88 ±0,1 to 5,79 ±0,1. However, the proportion of acid in the substrate in such an amount is quite high.

– In the case of using a substrate based on 20% of distillery grain, the longest release of hydrogen in the period from 3 (±0,05) to 6 (±0,05) hours of fermentation was achieved in the sample where the treatment was carried out with acid with a share of 1,5%. In this case, the pH of this sample during the synthesis of biohydrogen changed from 6,01 ±0,1 to 5,48 ±0.1, which is the most favorable for hydrogen-producing bacteria. However, the share of raw materials in such an amount creates technological difficulties in obtaining the supernatant at the stage of preliminary processing and conditions for an excessive increase in producers during microbial fermentation.

The total yield of biohydrogen ($V_{T.Y.}$) was analyzed during the fermentation of hydrolysates with minimum (4%) and maximum (20%) fractions of distillery grain, at which biohydrogen synthesis occurred in accordance with Figure 1.

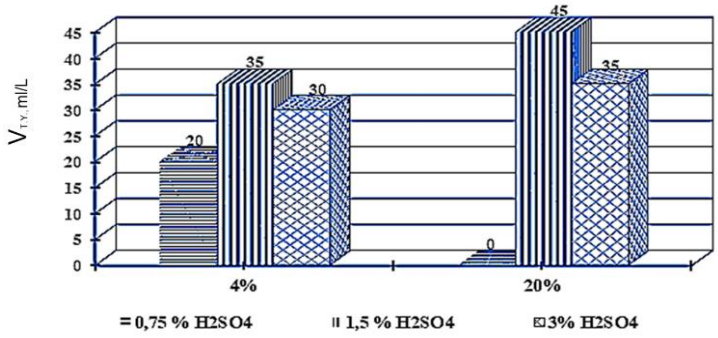


Figure 1 – Total hydrogen yield (ml/l) when using dilute sulfuric acid with fractions of 0,75%, 1,5% and 3% and wild-type *E. coli* for the treatment of substrates based on 4% and 20% fractions of distillery grain

Based on Figure 1, it can be stated that in the cases under consideration, the most effective treatment of the distillery grain was also with sulfuric acid at a concentration of 1,5%, where the maximum yield was 45±1,0 ml/l.

In this regard, the conditions for obtaining a hydrolyzate with a rational ratio of sugars were further studied by selecting the most suitable indicators for acid-hydrothermal treatment of distillery grain using wild-type *E. coli* for fermentation. In accordance with Figure 2, the dependence of the change in oxidation-reduction potential (ORP) and pH on the duration ($\tau_f$) of fermentation (at t = 37 °C, pH 7.5) of the substrate based on 10% of stillage using wild-type *E. coli* is presented.

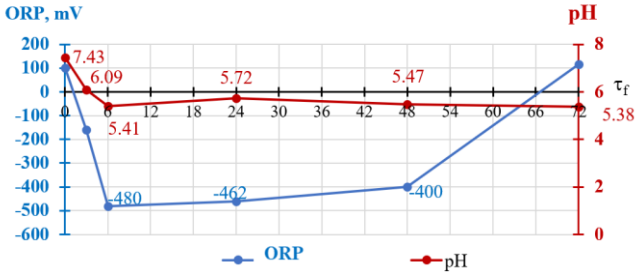


Figure 2 – Dependence of the change in oxidation-reduction potential (ORP) and pH on the duration ($\tau_f$) of microbial fermentation (at t=37°C, pH 7.5) of a substrate based on 10% of the fraction of distillery grain using wild-type *E. Coli*

As can be seen from Figure 2, the use of the established parameters of substrate pretreatment allowed us to significantly improve the results of exploratory experiments on biohydrogen synthesis. In particular, biohydrogen synthesis began at 3 o'clock and was prolonged to 48 hours of fermentation, while the total yield of biohydrogen was 116±1,0 ml / l. The principle underlying the use of acid-hydrothermal treatment is also that some bacteria are able to form spores under harsh conditions of temperature, pH, etc., and the formed endospores survive under extreme conditions of pretreatment, unlike non-spore-forming bacteria, which are suppressed.

**Conclusions**

When studying the effect of acid-hydrothermal treatment of distillery grain on the total yield of biohydrogen, it was found that the most effective proportion of sulfuric acid is 1,5% when using 10% of the raw material, where the highest yield of molecular hydrogen corresponded to 116±1,0 ml/l. Thus, the established conditions of acid-hydrothermal treatment of distillery grain contributed to an increase in H2 producers while suppressing H2 consumers during dark fermentation, and stimulating the yield of biohydrogen.

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## ҚАРАҢҒЫ АШЫТУ АРҚЫЛЫ ЛИГНОЦЕЛЛЮЛОЗАЛЫ БИОМАССАСЫНЫ ВАЛОРИЗАЦИЯЛАУ: МОЛЕКУЛАЛЫҚ СУТЕГІ БИОСИНТЕЗДЕУДІҢ ТИІМДІ ЖАҒДАЙЛАРЫН ЖАСАУ

*Лигноцеллюлозалы биомассаса (ЛБ) валоризациялау олардың қоршаған ортаға әсерін және адам денсаулығына қаупін азайту үшін маңызды. Қайта өңдеудің дәстүрлі әдістері, ең алдымен, лигноцеллюлозалы биомассаны органикалық заттардың құны жоғарылатылған өнім өндіру көзі ретінде емес, қалдық ретінде қарастыра отырып, қалдықтарды жоюға бағытталған. Сонымен қатар, экономикалық және экологиялық артықшылықтары бар құны жоғарылатылған өнімдер өндіретін үдерістерді әзірлеу қажет. Осыған байланысты, зерттеулер қараңғы ашыту кезінде сутектің шығымын арттыру мақсатында жұмыс параметрлеріне, алдын ала өңдеуге және микробтық ашытуға бағытталған. Бардаға негізделген субстраттардан жабайы типті E. coli көмегімен биосутек синтезі жүретін шикізаттың төменгі (4%) және жоғарғы (20%) концентрациялары анықталды. Спирттен кейінгі астық бардасына негізделген субстраттардағы шикізат пен қышқыл концентрациясының өзгеруі арқылы төмендететін қанттардың ұтымды қалыптасуының тиімді жағдайлары жасалды. Барданы қышқыл-гидротермиялық өңдеуідің биосутектің жалпы өнімділігіне әсерін зерттеу барысында күкірт қышқылының тиімді концентрациясы 1,5%, шикізаттың 10% үлесі пайдаланылатыны анықталды. Мұндай жағдайларда жабайы типті E. coli көмегімен молекулалық сутектің максималды шығымы 116 ±1,0 мл/л-ге жетті.*

***Түйін сөздер:*** *Валоризация, спирттен кейінгі барда, алдын ала өңдеу, қараңғы ферментациялау, қанттар, молекулалық сутек.*

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# ВАЛОРИЗАЦИЯ ЛИГНОЦЕЛЛЮЛОЗНОЙ БИОМАССЫ С ПОМОЩЬЮ ТЕМНОВОЙ ФЕРМЕНТАЦИИ: ВЫРАБОТКА ЭФФЕКТИВНЫХ УСЛОВИЙ БИОСИНТЕЗА МОЛЕКУЛЯРНОГО ВОДОРОДА

*Валоризация лигноцеллюлозной биомассой (ЛБ) имеет важное значение для снижения их воздействия на окружающую среду и снижения опасности для здоровья человека. Традиционные подходы к обработке вторичных сырьевых материалов в основном сосредоточены на утилизации, рассматривая лигноцеллюлозную биомассу как отход, а не как ценный ресурс для создания продуктов с добавленной стоимостью. В качестве альтернативы следует разработать процессы добавления стоимости отходам, производящим продукты с добавленной стоимостью, имеющие экономические и экологические преимущества. В этой связи, исследования были сосредоточены на рабочих параметрах, предварительной обработке и микробной ферментации с целью увеличения выхода водорода во время темной ферментации. Установлены верхние (4%) и нижние (20%) концентрации субстратов на основе барды, при котором происходит синтез биоводорода с применением E. coli дикого типа. Установлены условия для рационального формирования редуцирующих сахаров за счет вариации концентраций сырья и кислоты в субстратах на основе послеспиртовой зерновой барды. В ходе исследования воздействия кислотно-гидротермальной обработки барды на общий выход биоводорода было установлено, что оптимальная концентрация серной кислоты составляет 1,5%, при этом используется 10% доля сырья. При таких условиях был достигнут максимальный выход молекулярного водорода, равный 116 ±1,0 мл/л с применением E. coli дикого типа.*

***Ключевые слова:*** *Валоризация, послеспиртовая барда, предварительная обработка, темновая ферментация, сахара, молекулярный водород.*

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# НАССР ҚАҒИДАТТАРЫ НЕГІЗІНДЕ ТҮЙЕ СҮТІНЕН ЖАСАЛҒАН СҮТҚЫШҚЫЛДЫ ӨНІМНІҢ САПАСЫ МЕН ҚАУІПСІЗДІГІН БАСҚАРУ

***Аңдатпа:*** *Қазіргі уақытта отандық сүт және сүт өнімдерін өндіруші кәсіпорындарының басты мақсаты тұтынушыға басекеге қабілетті, жоғары сапалы және қауіпсіз сүт өнімдерін ұсыну болып табылады. Осы орайда қауіпсіз өнім өндірудің басты кепілі кәсіпорындарда ХАССП жүйесін енгізу болып табылады.*

*НАССР жүйесінің басты артықшылығы – азық-түлік өндірісінің бүкіл тізбегі бойынша кезең-кезеңмен бақылау арқылы қателіктерді анықтау, атап айтқанда алдын-алу және ескерту.*

*Зерттеу жұмысы ҚР СТ 1179-2003 «Сапа жүйелері. НАССР қағидаттарына негізделген тамақ өнімдерінің сапасын басқару. Жалпы талаптар» ұлттық стандарты негізінде жүргізілді. Бірінші кезеңінде түйе сүтінен жасалған сүтқышқылды өнім туралы бастапқы ақпараттар (құрамы, органолептикалық көрсеткіштері, сапасы мен қауіпсіздігін сипаттайтын көрсеткіштер, қолданылуы, қаптама түрі, сақтау және жеткізу шарттары) сипатталды және оны өндіру процессінің блок-схемасы құрастырылды. Екінші кезеңінде химиялық, биологиялық және физикалық тәуекелдерді талдау үшін түйе сүті мен одан жасалған өнімдердің сапасы мен қауіпсіздігіне қатысты нормативті құжаттар, ғылыми зерттеулер жүргізілді. Одан кейін анықталған қауіпті тәуекелдерді іске асырылу ықтималдығын бағалау жүзеге асырылды. Одан әрі, «Шешім қабылдау ағашы» құралының көмегімен зерттеу объектісінің технологиясында қауіпсіздікті басқаруға және сапасын арттыруға мүмкіндік беретін сыни бақылау нүктелері анықталды.*