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Biohydrogen production based on dark fermentation of molasses using escherichia coli

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Currently, dark fermentation is the most practically applicable for the implementation of biotechnological production of hydrogen. However, this process has certain limiting factors, since a significant part of the substrates are converted into various metabolic products, but not into H_2 . Therefore, it is necessary to develop optimal conditions for energy recovery in the form of gaseous molecular hydrogen. Various carbohydrate-containing raw materials for hydrogen production often require pretreatment before they can be used by microorganisms. Dilute acid pretreatment represents a promising way to increase biohydrogen production. However, during acid hydrolysis of carbohydrate-containing wastes, in addition to the released soluble sugars, inhibitors of enzymatic processing, such as furfural and 5-HMF, acetic and propionic acids, etc., can accumulate. In this regard, it is necessary to select the optimal conditions for the efficient production of biohydrogen. This study investigated the production of biohydrogen during the microbial fermentation of sugars in a dilute solution of a molasses-based acid hydrolyzate using Escherichia coli and a multiple mutant. The results of the experiments showed that molasses is a valuable product as a source of carbon and energy for microorganisms in the production of biohydrogen, as well as for the production of biomass for the further production of various products with high added value.

Keywords: carbon-containing waste, sugars, dark fermentation, biohydrogen, biomass.

Introduction

Hydrogen has become one of the alternative energy sources that can meet current energy demand and become a future carrier gas. It is considered a clean fuel because it does not emit CO_2 . The use of hydrogen is not limited to an energy source, but it can also be used as a raw material for the production of various

chemicals, hydrogenation of fats and oils in the food industry, and the production of methanol. About half of the hydrogen produced is used to make ammonia, which is again used to make fertilizers. Enormous amounts of hydrogen are also used in refineries to refine or convert heavier oils into lighter and more valuable products.

However, hydrogen is not available in nature and produced by costly chemical process, such as steam reforming of natural gas (Kim et al., 2011). Biological hydrogen production has attracted great attention because it can be performed at ambient temperature and pressure (Hallenbeck et al., 2012; Das and Veziroglu, 2008). Generally, biological hydrogen production could be divided into two categories: photo fermentation and dark fermentation (Panagiotopoulos et al., 2010).

Compared to photo fermentation, dark fermentative hydrogen production could be realized with advantages of higher hydrogen production rate and absence of light (Sagnak, et al., 2011). Moreover, dark fermentation could utilize a wide range of organic substrates (such as hexoses and pentoses) for hydrogen production (Urbaniec and Grabarczyk, 2009; Panagiotopoulos et al., 2015).

Hypothetically up to 12 mol of hydrogen can be obtained per mole of glucose, but there are no single metabolic pathways in nature that would allow this reaction. The theoretical yields of hydrogen from dark fermentations depend on the type of organisms that are used in fermentation (Mathews and Wang 2009).

Escherichia coli is the most extensively utilized strain for hydrogen production and metabolic engineering studies [1, 2]. There are four hydrogenases in the E. coli genome, hydrogenase 1, hydrogenase 2, hydrogenase 3, and hydrogenase 4. These hydrogenases possess different functions as comprehensively described beforehand [3]. In addition, there are some of characterized genes in E. coli, which are responsible for hydrogen production [4]. Thus, the mechanism of hydrogen metabolism is still under investigation.

Formulation of the problem. Low process yield and incomplete conversion of organic biomass are the two main bottlenecks for the commercial production of dark fermented biohydrogen (Gomez et al., 2011; Ren et al., 2011). Earlier, some reasons were put forward for which the industrial production of biohydrogen is not yet feasible. These problems include ensuring the sustainability of production from various substrates, determining the conditions for inhibition of the process and their avoidance, optimizing the overall process parameters, and, finally, the safety and economic problems associated with the creation of a hydrogen storage system. However, dark enzymatic biohydrogen production can be increased through the selection of an appropriate substrate, inoculum enrichment strategies, and optimal substrate pretreatment. Therefore, research becomes relevant in order to establish optimal operating conditions in the dark enzymatic production of H_2 from a specific type of raw material.

Several studies have also examined optimal operating conditions (e.g., culture pH, temperature, substrate utilization, and inoculum enrichment) to maximize H_2 production in DF (Cappai et al., 2014; De Gioannis et al., 2013; Ghimire et al., 2015a; Luo et al., 2010; Wang and Wan, 2011; Wong et al., 2014). However, these methods inevitably generate several groups of toxic inhibitors and have an

inhibitory effect on fermentation [5, 6].

In this regard, the object of this work was to study the isolation of biohydrogen molecules from molasses by dark fermentation.

Dark fermentation characteristic. Biohydrogen production through dark fermentation is preferred over other biological processes because of its unique properties such as rapid cell growth, independence from light and, most importantly, an economical approach because it can recycle waste (Hallenbeck and Benemann 2002; Kotay and Das 2008; Nath and Das 2004). It involves anaerobic digestion of pyruvate by enzymatic mechanisms in microorganisms that ferment to digest carbohydrates into biohydrogen.

Dark fermentation refers to the decomposition of organic substrates by anaerobic bacteria in an environment devoid of light and oxygen to produce biohydrogen [7, 8].

The breakdown and transformation of complex polymers such as carbohydrates into biohydrogen occurs through a series of biochemical reactions. Carbohydrate-rich materials are initially hydrolyzed to sugar molecules either biologically or using pretreatment technologies [9, 10].

Pretreatment can be applied to both seed and substrates [11]. For pretreatment, in most cases, both dilute acid hydrolysis and concentrated acid hydrolysis are used. The dilute acid process is carried out at high temperature and pressure, while the concentrated acid process uses relatively mild conditions with much longer reaction times. Pretreatment of seed material is used to enrich H_2 producers and suppress H_2 consumers [12-15].

The purpose of substrate pretreatment is to destroy the lignin insulation of lignocellulosic materials, release cellulose molecules into solution, destroy the crystalline structure of cellulose and promote its retention by depolymerization to enhance hydrolysis and biohydrogen production [16-19]. In addition to these positive aspects, pretreatment of lignocellulosic substances also leads to the formation of toxic by-products such as phenolic compounds, furan derivatives and weak acids, which can inhibit the production of dark enzymatic biohydrogen [20-24].

Furfural is formed as a result of the decomposition of xylose at high temperature and pressure, while the formation of HMF occurs as a result of the decomposition of pentose. Further decomposition of furfural leads to the formation of formic acid, while thermal decomposition of HMF with an acid leads to the synthesis of formic and levulinic acid [25-27].

Research shows that acid pretreatment as well as heat treatment are more effective than chemical inhibition, aeration and base treatment to completely suppress methanogenic activity (Zhu and Beland 2006).

Temperature is one of the main factors affecting the formation of biohydrogen. The mesophilic temperature is considered inexpensive and easily controlled on a large scale. The production of biohydrogen in a mesophilic environment uses less energy.

Temperature plays an important role. Although high temperatures tend to solubilize the substrate, prolonged exposure can denature cellular proteins, but may limit the intervention of hydrogen consumers. Thus, an optimum

temperature of 30-35° C has been described for efficient biohydrogen production by increasing cell growth and ultimately a product of interest (Cardoso et al., 2014; 450 22 Bioprospecting of Microbes for Biohydrogen Production: Current Status and Future Challenges Lee et al. 2008). In contrast, other reports suggest that the optimum temperature is above 45° C because the solubility of hydrogen at high temperatures is low (Hallenbeck and Benemann 2002). Various types of crops are used in biohydrogen fermentation to biodegrade various substrates. E. coli is one of the best microorganisms for hydrogen production because genetic manipulation (including transcriptomics, P1 phage transduction, and use of the KEIO/ASKA library) is developed as well as the biochemistry of many metabolic pathways for enhanced hydrogen production is understood (Blattner et al. 1997). In E. coli, hydrogen is generated from formate by a formate hydrogen lyase (FHL) system consisting of hydrogenase 3 (hycABCDEFGHI) (Bagramyan and Trchounian, 2003) and formate dehydrogenase-H (fdhF) (Axley et al. 1990). HycA inhibits FHL activity (Bagramyan and Trchounian 2003), while FhlA activates FHL function (Schlensog et al. 1994). Hydrogen is consumed by E. coli hydrogenase 1 (hyaABCDEF) (Forzi and Sawers 2007) and hydrogenase 2 (hybOABCDEFG) (Forzi and Sawers 2007).

Materials and methods

Pre-treatment of raw materials and preparation of the substrate. For testing, a substrate was prepared with 4 and 10% molasses concentrations. All samples passed the following types of tests: treatment with sulfuric acid (H_2SO_4) with concentrations of 0.75% and 1.5%, autoclaving at a temperature of 121° C, purification from impurities by filtration and centrifugation, pH calibration of the medium to 7.5 units. with the use of KOH and K_2NPO_4 .

Night culture preparation. Overnight cultures were prepared using wild-type and multiple mutant E. coli BW25113 cultured in peptone medium under anaerobic conditions at 37° C for 18-20 hours.

The introduction of the night culture into the substrate. On the prepared substrates of molasses were introduced overnight cultures with a ratio relative to the total mass of the sample. Further, the obtained samples were subjected to microbial fermentation, where an anaerobic condition and a constant temperature (37° C) of the chamber were also provided.

Measurement of oxidation-reduction potential (ORP) and pH. The ORP of the bacterial medium was determined using a pH/ORP Meter HI 2211 with a pH electrode HI 3131 (HANNA Instruments, Portugal). The pH of the substrate measured by a pH meter HI 3220 with a pH selective electrode HI 1131 (Hanna Instruments, Portugal).

Research results and discussion.

The evolution of molecular hydrogen was studied by measuring the oxidation-reduction potential (ORP) of substrates from molasses with varying conditions

Table 1.
The results of the evolution of molecular hydrogen during dark fermentation of substrates based on molasses (different concentrations of dilute sulfuric acid).

Time (h)	Indicators	Fermentation with wild-type E. coli					Fermentation with E. coli mutant	
		H_2SO_4-0.75%, (pH calibration with KOH)	H_2SO_4-1.5% (pH calibration with KOH)	H_2SO_4-1.5% (pH calibration with KOH)	H_2SO_4-0.75% (pH calibration with K_2HPO_4)	H_2SO_4-0.75% (pH calibration with K_2HPO_4)	H_2SO_4-1.5% (pH calibration with KOH)	H_2SO_4-0.75% (pH calibration with K_2HPO_4)
		Molasses concentration 4%	Molasses concentration 4%	Molasses concentration 10%	Molasses concentration4%	Molasses concentration 10%	Molasses concentration 10%	Molasses concentration 10%
1	2	3	4	5	6	7	8	9
0	ORP mV	-13	35	-20	-235	-243	-63	-233
	pH	6.83	7.11	6.76	7.45	7.32	6.04	7.34
3	ORP mV	-27	-63	-56	-274	-260	-54	-255
	pH	6.18	5.29	5.84	7.39	7.34	5.92	7.32
6	ORP mV	-422	-477	-461	-357	-252	-92	-384
	pH	5.27	4.9	5.21	7.40	7.35	5.93	7.19
24	ORP mV	-443	-399	-464	-600	-479	-48	-578
	pH	4.78	4.46	4.65	7.23	7.15	5.83	7.16
48	ORP mV	48	104	-15	-550	-438	-24	-617
	pH	4.54	4.27	4.46	7.17	7.01	5.86	7.05

Continue of Table 1.

1	2	3	4	5	6	7	8	9
72	ORP mV			48	-530	-457	-50	-585
	pH			4.41	7.22	7.10	5.84	6.93
96	ORP mV			72	-556	-546	-212	-583
	pH			4.34	7.1	6.95	5.74	6.86
120	ORP mV				-470	-520		-569
	pH				7.18	7.00		7.00
144	ORP mV				-413	-417		531
	pH	Further	Further	Further	7.18	7.03	Further	6.98
168	ORP mV	measurements	measurements	measurements	-463	-572	measurements	-355
	pH	are paused	are paused	are paused	7.23	7.02	are paused	7.13
192	ORP mV				-200	-552		
	pH				7.34	6.98		
216	ORP mV				Further	-549		
	pH				measurements	6.97		
240	ORP mV				are paused	-173		
	pH					6.98		

for preparing raw materials and carrying out microbial fermentation. Wild-type E. coli BW25113 and its multiple mutant were used as hydrogen-producing microorganisms. The dynamics of the change in the pH of the medium in all experimental samples was also studied. All ORP and pH measurement results are shown in Table 1.

Fermentation using wild type E. coli BW25113. In all experimental samples, where during the preliminary treatment, the pH was adjusted with K_2HPO_4 , a slight decrease in pH was observed, in the range from 7.5 to 6.98. In samples where the pre-treatment pH was adjusted with KOH, a significant decrease in pH was observed. For example, (1) in samples with 4% molasses concentration (0.75% H_2SO_4) at 48 hours, the pH dropped to 4.54, (2) in samples with 4% molasses concentration (1.5% H_2SO_4) at 48 hours, the pH dropped to values of 4.66; in samples with a 10% concentration of molasses (1.5% H_2SO_4) at 96 hours, the pH dropped to 5.43.

In addition, as can be seen from Table 1, column 3: in samples with a 4% concentration of molasses, where diluted sulfuric acid with a concentration of 0.75% was used for (1) pretreatment and for (2) pH regulation – KOH, at the 6th hour of measurements, the release of hydrogen molecules was observed and continued until the 24th hour. At the same time, in the same samples (column 4 of Table 1) with a 4% concentration of molasses, but for preliminary treatment where dilute sulfuric acid with a concentration of 1.5% was used, the release of hydrogen molecules was observed only on the 6th hour of measurements. A similar dynamics of the release of hydrogen molecules was observed (6-24 hours) in samples (column 5 of Table 1) with a 10% concentration of molasses, where (1) was also used for preliminary treatment - diluted sulfuric acid with a concentration of 1.5% and (2) for pH regulation – KOH.

Better results were obtained in the samples (column 6 of Table 1), where the pH was adjusted with K_2HPO_4 , for example (1) the evolution of hydrogen molecules began at the 24th hour of measurements and continued until the 168th hour in samples with a 4% concentration molasses (0.75% – H_2SO_4)).

The best results were achieved in samples (column 7 of Table 1) with a 10% molasses concentration, where 0.75% H_2SO_4) was used in the preliminary treatment and the pH was adjusted with K_2HPO_4 at the 24th hour of measurements, the evolution of hydrogen molecules was observed and continued up to 216th hour.

Fermentation with multiple mutant E. coli BW25113. In all experimental samples, where during the preliminary treatment the pH was adjusted with K_2HPO_4 , a slight decrease in pH was observed, in the range from 7.5 to 7.0. In all experimental samples of molasses hydrolyzate, where pH was regulated with KOH, a significant decrease in pH was observed, for example, in a sample (1.5% H_2SO_4) and KOH) with a 10% molasses concentration at 96 hours, the pH value dropped to 5.74.

In order to compare with the best results obtained with E. coli BW25113 wild type, samples with a 10% concentration of molasses were selected. In all samples (column 8 of Table 1) with a 10% concentration of molasses, where 1.5% H_2SO_4) was used in the preliminary treatment and the pH was adjusted with KOH, no

hydrogen evolution was detected.

Nevertheless, in samples (column 9 of Table 1) with a 10% concentration of molasses, where 0.75% H_2SO_4) was used in the preliminary treatment and the pH was adjusted with K_2HPO_4, the evolution of hydrogen molecules was observed at the 24th hour of measurements and continued up to 168th hour.

In the course of the experiments, the cumulative yield of hydrogen in the samples was also studied, where the best results on the evolution of molecular hydrogen were obtained. The hydrogen yield was 1640 ml/l in experimental samples, where molasses substrates were fermented using wild type E. coli BW25113. The highest value of 1690 ml/L of biohydrogen yield was recorded in samples where molasses substrates were fermented using multiple mutant E. coli BW25113.

The data obtained indicate the relevance of further study on the influence of various factors on the production of hydrogen from molasses.

Conclusion

On the basis of experimental data, it can be stated that the optimal conditions for pretreatment and fermentation of molasses with the use of various hydrogen-forming bacteria have been selected to intensify the release and yield of biohydrogen. Biohydrogen production using Escherichia coli continues to be a valuable model that can provide useful information to understand the pathways for hydrogen production from various energy sources. It should also be noted that as a result of bioconversion, biomass is reproduced, which is a medium rich in various organic substances, for further secondary and full use to obtain other products with high added value.

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