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Enhanced biohydrogen production from beverage industrial wastewater using external nitrogen sources and bioaugmentation with facultative anaerobic strains

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In this work biohydrogen generation and its improvement possibilities from beverage industrial wastewater were sought. Firstly, mesophilic hydrogen fermentations were conducted in batch vials by applying heat-treated (80°C, 30 min) sludge and liquid (LB-grown) cultures of *Escherichia coli* XL1-Blue/*Enterobacter cloacae* DSM 16657 strains for bioaugmentation purposes. The results showed that there was a remarkable increase in hydrogen production capacities when facultative anaerobes were added in the form of inoculum. Furthermore, experiments were carried out in order to reveal whether the increment occurred either due to the efficient contribution of the facultative anaerobic microorganisms or the culture ingredients (in particular yeast extract and tryptone) supplied when the bacterial suspensions (LB media-based inocula) were mixed with the sludge. The outcome of these tests was that both the applied nitrogen sources and the bacteria (*E. coli*) could individually enhance hydrogen formation. Nevertheless, the highest increase took place when they were used together. Finally, the optimal initial wastewater concentration was determined as 5 g/L.

[**Key words:** Beverage wastewater; Bioaugmentation; *Escherichia coli* XL1-Blue; *Enterobacter cloacae* DSM 16657; External nitrogen source]

Due to the climate issues and concerns about the current fossil-based energy systems, a remarkable and world-wide progress in the research of environmental-benign and renewable energy carriers has started in the last couple of decades. Up to now, a large variety of alternative biofuels have been investigated and biologically produced hydrogen is considered as a possible solution for the future because of its unique characteristics (1). Among the different approaches to generate biohydrogen, the so-called dark fermentative way seems to be a potential candidate for large-scale applications from economical and practical point of views (2,3). During dark fermentation, hydrogen is formed from different organic compounds via the metabolic activity of specific microorganisms. The process is affected by a number of factors such as temperature, pH, carbon and nitrogen sources and the composition of the microbial consortia (4,5). Recently, enormous efforts have been put to make dark fermentation even more competitive, however, mostly simple, pure substrates were used and therefore the utilization of industrial waste streams still receives particular interest (6).

In general, the bioconversion of such complex materials into hydrogen requires a good cooperation of diverse microbial consortia that can usually be found in sewage sludge, anaerobic digesters, etc. However, sludge pretreatment is mostly compulsory in order to suppress the activity of methanogenic species or in other words, to help the selective growth of the potential hydrogen producers. The range of proven pretreatment techniques includes heat-shock, chemical agents (chloroform), acidic/alkali pretreatment, aeration, freezing and thawing, and so on (6). Among them, heat-pretreatment is definitely the most widely and routinely employed since it is relatively fast, easy to conduct and efficient (7). However, its drawback is that it could inhibit some of the reliable hydrogen producers, as well (3,6). In most cases when heat treatment is applied it could observed that the mixed microbial community was strongly dominated by spore-forming species (e.g., *Clostridia*) and non sporulative hydrogen fermenting bacteria such as facultative anaerobes like the members of *Enterobacteriaceae* could not survive. This reduction in the microbial diversity of the mixed culture might bring disadvantages in process efficiency. Consequently, the bioaugmentation of heat-treated sludge with facultative anaerobic strains might result in the improvement of hydrogen production. Moreover, in studies when their pure cultures were used some significant achievements have been published, however, mostly with simple substrates (8). Hence, investigating the possibility related to bioaugmented hydrogen production by applying various facultative anaerobes could be an interesting field of research. Up to authors' best knowledge, no biohydrogen research has been conducted on industrial wastes using bioaugmented, heat-treated inocula. Therefore, in this study, bioaugmentation of mesophilic hydrogen fermentation by employing two facultative anaerobic organisms, *Escherichia coli* XL1-Blue and *Enterobacter cloacae* DSM 16657 was carried out from

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beverage industrial wastewater under different culture conditions. Alongside these tests, the effect of external nutrient (tryptone, yeast extract) addition was also sought since previously it was demonstrated that supplementation with such nitrogen sources led also to enhanced hydrogen generation (9).

MATERIALS AND METHODS

Bacterial source *E. coli* XL1-Blue and *E. cloacae* DSM 16657 strains were supplied by University of Pannonia, Veszprem, Hungary as a part of a scientific program with Feng Chia University, Taiwan. Sewage sludge was obtained from a local water treatment plant Taichung, Taiwan. The collected sludge was stored in refrigerator at 4°C and heat-pretreated at 80°C for 30 min prior to use. In accordance with our previous paper (10), the method of optical density at 620 nm (OD_{620}) was employed to follow the biomass growth in the inoculum of the pure cultures mentioned above. LB media was used to prepare the inoculum of the facultative anaerobic bacteria mentioned and their overnight cultures were applied for bioaugmentation purposes.

Beverage industrial wastewater The wastewater feedstock was collected from a beverage industrial company located in central Taiwan. The characteristics of the beverage wastewater (BWW) were pH 2.6–3.4, 760–900 g COD/L and total reducing sugar of 660–750 $g_{(glucose\ equivalent)}$/L. From that, a 40 g COD/L stock solution was prepared and used in the experiments. The wastewater was kept at 4°C in order to avoid any biological changes and during the experiments the 40 g COD/L stock solution was diluted to get the desired substrate (COD) concentrations.

Hydrogen fermentation Batch fermentations were carried out in vials having a total capacity of 225 mL, with an effective working volume of 160 mL was used. Slightly acidic initial pH was selected and adjusted to 6.5 (6,11). The bottles were purged with argon gas for 10 min in order to ensure anaerobic conditions, subsequently sealed and placed in a reciprocal air-bath shaker at 150 rpm with temperature control at 37°C. The nutrient solution used in this study was slightly different from the Endo formulation (12) and contained the following ingredients (mg/L): 125 K_2HPO_4, 100 $MgCl_2 \cdot 6H_2O$, 15 $MnSO_4 \cdot 6H_2O$, 25 $FeSO_4 \cdot 7H_2O$, 5 $CuSO_4 \cdot 5H_2O$, and 0.12 $CoCl_2 \cdot 5H_2O$. The volume of biogas produced was measured by air-tight glass syringe and composition was determined periodically by gas chromatography as described elsewhere (13,14). Fermentations were terminated when no further gas production could be observed. All the measurements were carried out in triplicates and results are given as their mathematical averages.

Modified Gompertz equation Modified Gompertz equation (Eq. 1) was used to get the kinetic parameters such as hydrogen production potential (P), maximum hydrogen production rate (R_m) and lag phase time (λ) under different experimental conditions. The software details are Sigma plot software 10.0 (Systat Software Inc., USA).

$$H(t) = P \cdot \exp\left\{ -\exp\left[\frac{R_m \cdot e}{P}(\lambda - t) + 1\right]\right\} \quad (1)$$

where $H(t)$ represents the cumulative hydrogen production (mL); P is the hydrogen production potential (mL); R_m is the maximum hydrogen production rate (mL/h); e is 2.718; λ is the duration of lag phase (h) and t is the cultivation time (h). Hydrogen production rate (HPR; L H_2/L-d) was defined as R_m value divided by the working volume of the reactor and multiplied with a day in hours (24 h). Hydrogen yield (HY; mL H_2/g COD added) was calculated as the cumulative hydrogen production (mL) divided by the substrate (COD) added.

PCR amplification of genomic DNA Total genomic DNA from the enriched mixed cultures was extracted by using the Blood & Tissue Genomic DNA Extraction Miniprep System (Viogene, Taiwan) following the manufacturer's instructions. PCR primers employed for the amplification of 16S rDNA was, the eubacterial primer set (forward primer Eub968f with GC clamp and reverse primer Univ1392r) (15). The PCR amplification and DGGE analysis were performed in accordance with the method described in our previous study (14).

RESULTS AND DISCUSSION

Hydrogen fermentation from beverage wastewater using heat-pretreated, bioaugmented sewage sludge The effect of bioaugmentation on hydrogen production was studied by the addition of facultative anaerobic strains belonging to *Enterobacteriaceae*, namely *E. coli* and *E. cloacae*. These microbes were proven to be robust and efficient hydrogen producers by different scientists. For example, Kumar and Das (16) used *E. cloacae* for hydrogen production and attractive yields and production rates were reported from various substrates, e.g., glucose, fructose, sucrose, xylose, cellobiose. In another publication, Ghosh and Hallenbeck (17) applied *E. coli* for biotechnological hydrogen generation from substrates covering disaccharides (e.g., lactose, sucrose, maltose), monohexoses (e.g., glucose, fructose, galactose) and monopentoses (e.g., xylose, arabinose), as well. It was revealed that these microorganisms could sufficiently transform this wide range of carbon sources to hydrogen and therefore, *E. cloacae* and *E. coli* can be considered as promising bacteria for viable hydrogen fermentation (16,17). However, still only a limited knowledge is available on hydrogen production from complex substrates using mixed cultures and the above mentioned pure cultures, as well. Consequently, in this study, beverage industrial wastewater was used as carbon source for bioaugmented hydrogen generation.

Firstly, 5 g COD/L was chosen as initial BWW substrate concentration and 5 various experimental runs were carried out according to Table 1. The obtained fermentation profiles are depicted in Fig. 1. As it can be seen in Table 1, *E. coli* XL1-Blue, *E. cloacae* DSM 16657 and their mixture (1:1 ratio) were added as overnight cultures grown in liquid Luria–Bertani (LB) media. Based on spectrophotometric calibration curves (OD_{620}), 40 mL was calculated as the required inoculums size in order to ensure an initial cell density of 0.1 g dry cell weight/L in the broth.

Evaluating the results in Fig. 1, it can be concluded that addition of liquid cultures regardless of the bacteria could significantly affect both HPR and HY, which could possibly be attributed to two different reasons. On one hand, it might occurred due to the efficient contribution of the applied strains. On the other hand, it could be the outcome of the nutrients (e.g., yeast extract, tryptone) supplementation along with the bacteria. These materials are the main ingredients of LB medium, which was employed to prepare the overnight inocula of the pure cultures, and could reportedly enhance the hydrogen production efficiency (9). Furthermore, based on Table 2 it can be pointed out that the highest improvement could be achieved when the heat-treated sludge was bioaugmented by *E. coli* XL1-Blue, followed by mixture of *E. coli* XL1-Blue and *E. cloacae* DSM 16657 (50:50%) and the *E. cloacae* DSM 16657 alone.

Hydrogen generation from beverage wastewater using pure cultures of *E. coli* XL1-Blue and *E. cloacae* DSM 16657 Measurements were conducted with the pure cultures of the facultative anaerobic bacteria (Table 3) in order to seek whether the *E. coli* XL1-Blue, *E. cloacae* DSM 16657 or their mixture possess the ability of producing hydrogen from the beverage waste water or not. The applied initial wastewater COD concentration and cell density were adjusted as previously to 5 g COD/L and 0.1 g dcw/L, respectively.

The results are shown in Fig. 2, where it can be seen that hydrogen could be formed quite satisfactory, however a bit surprisingly, the progress curves in Fig. 2 were remarkably different compared to those in Fig. 1. From the data listed in Table 2 it can be drawn that biohydrogen fermentation employing these facultative anaerobe strains can be characterized by a prolonged lag phase in the following order: *E. cloacae* DSM 16657 < *E. coli* XL1-Blue: *E. cloacae* DSM 16657 (50:50%) < *E. coli* XL1-Blue. Furthermore, unlike

TABLE 1. The composition of experimental trials for Fig. 1.

	1	2	3	4	5
PTS (mL)	20	20	20	20	20
BWW (mL)	–	20	20	20	20
E. coli in LB (mL)	–	–	40	–	20
E. cloacae in LB (mL)	–	–	–	40	20
NS (mL)	5	5	5	5	5
LB (mL)	–	–	–	–	–
DI water (mL)	135	115	75	75	75
Total volume (mL)	160	160	160	160	160

PTS, pretreated sludge; Bww, beverage wastewater; NS, nutrient solution; LB, Luria–Bertani media.