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Comparative analysis of hydrogen-producing bacterial biofilms and granular sludge formed in continuous cultures of fermentative bacteria

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ABSTRACT

A system for biohydrogen production was developed based on long-term continuous cultures grown on sugar beet molasses in packed bed reactors. In two separate cultures, consortia of fermentative bacteria developed as biofilms on granitic stones. In one of the cultures, a granular sludge was also formed. Metagenomic analysis of the microbial communities by 454-pyrosequencing of amplified 16S rDNA fragments revealed that the overall biodiversity of the hydrogen-producing cultures was quite small. The stone biofilm from the culture without granular sludge was dominated by *Clostridiaceae* and heterolactic fermentation bacteria, mainly *Leuconostocaeae*. Representatives of the *Leuconostocaeae* and *Enterobacteriaceae* were dominant in both the granules and the stone biofilm formed in the granular sludge culture. The culture containing granular sludge produced hydrogen significantly more effectively than that containing only the stone biofilm: 5.43 vs. 2.8 mol H_2/mol sucrose from molasses, respectively. The speculations that lactic acid bacteria may favor hydrogen production are discussed.

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1. Introduction

Hydrogen is both a valuable energy carrier and a feedstock for various branches of the chemical industry. Conventional methods of hydrogen production based on fossil fuels are energy-expensive and cause environmental pollution. Therefore, there is great interest in bacterial dark fermentation as an alternative method of hydrogen production. However, the low hydrogen yield and the generation of large quantities of non-gaseous organic products remain key problems of dark fermentation. The theoretical maximum hydrogen yield during dark fermentation is 4 mol H_2/mol glucose (~33% substrate conversion), but the actual yield is only 2 mol H_2/mol glucose (~17% conversion). Currently, many investigations are focused on both improvement of the hydrogen yield during fermentation and the combination of dark fermentation with other processes like methanogenesis, photofermentation or microbial electrolysis cells to achieve more effective substrate utilization (Li and Fang, 2007; Hallenbeck and Ghosh, 2009; Lee et al., 2010). In addition, there is increasing interest in the production of short-chain fatty acids like butyric acid in microbial fermentation processes, as bio-based natural ingredients for the food, cosmetics, and pharmaceutical industries (Jiang et al., 2009).

Biohydrogen fermentations may be carried out in different types of batch, continuous or semi-continuous bioreactors, where mixed microbial consortia develop. In the most effective systems, consortia are selected for growth and dominance under non-sterile conditions and usually show high stability and resistance to transient unfavorable changes in the bioreactor environment. Depending on the bioreactor type and growth conditions, the consortia form various structures ensuring the retention and accumulation of the active biomass. These include microbial-based biofilms and macroscopic aggregates of microbial cells such as flocs and granules (Skiadas et al., 2003; Hallenbeck and Ghosh, 2009). A biofilm is a complex coherent structure of cells and cellular products, like extracellular polymers, immobilized on a substratum that can be a static solid surface (static biofilms) or suspended carriers (particle-supported biofilms). Granules are conglomerates of microbial cells adhering to each other and forming dense, usually spherical, structures of ~0.14–5 mm in diameter. The extracellular polymeric matrix is an abundant granular component that is responsible for the structure and function of granules. Granules are thought of as a special type of biofilm formed under conditions where solid surfaces are absent. Shear stress, caused by the operational hydrodynamic conditions in bioreactors, is thought to be one of the most important factors promoting granule formation (Skiadas et al., 2003; Campos et al., 2009).

Many studies on hydrogen-producing biofilms, sludge, flocs or granules have focused on the performance and efficiency of the

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entire process, but they have lacked deep analysis of the microbial communities (Ren et al., 2006; Li et al., 2007; Baghchehsaraee et al., 2009).

A good understanding of the structure of hydrogen-producing microbial communities is vital for attempts to optimize H_2 production. The techniques most commonly used for analyzing the diversity of hydrogen-producing microbial communities are polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and direct sequencing of DGGE bands (Fang et al., 2002; Kim et al., 2006; Hung et al., 2007; Ren et al., 2007), or cloning and sequencing of the DNA from these bands (Li et al., 2006; Wu et al., 2006). However, these strategies often lead to an underestimation of the true bacterial diversity as a result of co-migration of different sequences during electrophoresis and concentrating only on the most prominent DGGE bands. Examinations of the biodiversity within hydrogen-producing consortia are mainly based on 16S rDNA gene sequence analysis.

Recently developed high-throughput ultrafast sequencing technologies are being increasingly used to generate the raw data for such analyses (Jaenicke et al., 2011).

This report describes a comparative analysis of the biodiversity in biofilms and granules formed by hydrogen-producing bacteria in two continuous flow cultures grown on sugar beet molasses in packed bed reactors. 454-Pyrosequencing was used to generate sequence data for the phylogenetic characterization of the hydrogen-producing microbial communities. The relationship between bacterial consortium structure and the efficiency of hydrogen production was also examined.

2. Methods

2.1. Seed sludge and feed composition

The seed sludge inoculum was obtained from a eutrophic, meromictic lake, as described previously (Sikora et al., 2011). The cultivation medium was M9 medium supplemented with trace elements (Sikora et al., 2011) and molasses from Dobrzelin Sugar Factory (Poland). The organic loading rate (OLR) was 54 g molasses COD (chemical oxygen demand)/L. The medium was sterilized by boiling and saturated with a stream of pure N_2 or a mixture of N_2:CO_2 (80:20) (Air Products, Poland).

2.2. Enrichment technique and history of cultures

A 3 L-packed bed reactor (PBR) made of plexiglass was filled with cultivation medium containing molasses and inoculated anaerobically with a 30-ml sample of lake bottom sediment. Granitic stones (Ø2–3 cm) were put into the bioreactor to act as a solid phase to permit bacterial biofilm development on their surface. The culture was incubated at room temperature for 17 days. The medium flow was then switched on and the bacterial culture was maintained for 125 weeks at room temperature. The hydraulic retention time (HRT) was 10–18 h. The working volume of the bioreactor was 1.5 L. Bacterial biofilm on granitic stones and granular sludge were formed in the bioreactor, the latter was observed from the 4th week of cultivation. After the 12th week of cultivation the granules were no longer observed. Subsequently, the bacterial biofilm was the only structure formed by bacteria in the bioreactor. The culture was renewed regularly by removing the excess biomass every 10 days when granular sludge was present and every 4–6 weeks after the granules had disappeared. The presented results describing the culture containing the biofilm come from the period after the disappearance of the granular sludge.

Two samples of the culture described above, taken in the 9th and 110th weeks of cultivation, were mixed with an equal volume of 30% glycerol and stored frozen at −80 °C. These samples served as the inoculum for the next culture. They were added to the PBR filled with cultivation medium and granitic stones. The medium flow was switched on after 13 days of incubation at room temperature. This second culture was maintained for 52 weeks. The HRT was 10–18 h. After 2 weeks of cultivation, granular sludge was formed in the bioreactor and this remained until the 52nd week. The presented results describing the culture containing the biofilm and granules come from the period after the 3rd week of cultivation.

2.3. Analytical methods

To enumerate bacteria in the bioreactor, a three-tube most probable number (MPN) assay was used. Samples of culture taken from the bioreactor were diluted in LB medium (1% tryptone, 0.5% yeast extract and 0.5% NaCl) containing 0.5% glucose. A 10-fold dilution series was prepared in an anaerobic chamber (Coy Laboratory Products, USA) and incubated at 37 °C for 1 day. MPN values were calculated using McCrady's table as described before (Sikora et al., 2011). The pH of the effluent was measured using a standard pH meter (WTW, inoLab). The COD of the medium and the effluent was determined using the dichromate method according to ISO 6060:1989, water quality – determination of the chemical oxygen demand. Before COD determination, the effluent was centrifuged to remove bacterial cells.

At selected time points during each cultivation experiment, the fermentation gas was collected in a gas pipette. A 250-cm^3 gas pipette was filled with saturated KCl solution and connected to the bioreactor via a gas-tight junction. The total rate of gas production was determined and levels of H_2, CO_2, and CH_4 were subsequently quantified by gas chromatography (Shimadzu GC-14B, Carboxen 1010 PLOT column, TCD detection). In selected gas samples, levels of H_2S, short-chain carboxylic acids, and NH_3 were also determined (Hewlett Packard 6890, pre-esterification of acids, polar 0.3-micrometer capillary column, and FID or AED detection).

At the same selected time points, samples of the cultivation medium and effluent were collected, centrifuged twice and the concentrations of carbohydrates (sucrose, glucose and fructose), short-chain fatty acids and ethanol in these supernatants were determined using, respectively, high performance liquid chromatography (HPLC) and refractometric detection (Waters HPLC system: Waters 2695 – Separations Module, Waters 2414 – Refractive Index Detector, and Sugar Pak column 300 × 6.5 mm with guard column); HPLC and photometric detection (Waters HPLC system as above, Waters 2996 – Photodiode Array Detector, and Aminex HPX-87 H column 300 × 7.8 mm with guard column); and gas chromatography and flame-ionization detection (Hewlett Packard 6890, autosampler headspace – Hewlett Packard 7694E, polar 1.0-μm capillary column and FID). HPLC conditions for determination of carbohydrates were: column temperature of 90 °C; flow rate, 0.5 ml/min; injected volume, 10 μl; mobile phase, water. HPLC conditions for determination of organic acids were: column temperature of 35 °C; flow rate, 0.6 ml/min; injected volume, 10 μl; mobile phase, 4 mM sulfuric acid.

2.4. Total DNA isolation

Total DNA was extracted from bacterial communities in the bioreactors by a combination of a standard procedure for soil, using mechanical matrix disruption, and repetitive freeze–thawing, as described previously (Tomczyk-Żak et al., 2011), with slight modifications as described below. Fifteen stones were taken aseptically from the bioreactor and samples of biofilm were collected from them with a sterile toothbrush and by rinsing with 0.9% NaCl. The obtained particulate suspension was centrifuged and the

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