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Integration of purple non-sulfur bacteria into the starch-hydrolyzing consortium

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ABSTRACT

Purple non-sulfur bacteria *Rhodobacter sphaeroides* N7 was integrated into the heterotrophic starch-hydrolyzing consortium containing *Clostridium butyricum* as the main component. The resulting light-dependent consortium with Bchl ~9 μg/ml was maintained during 12 months of regular transfers. The protein content in this consortium was four-fold higher compared to the heterotrophic consortium, suggesting that purple bacteria became its important component. Under illumination, the starch hydrolysis by the light-dependent consortium (as opposed to heterotrophic consortium) resulted in the absence of VFAs, hydrogen production being quite similar and residual hexose higher. Fermentation of glucose (unlike to starch) by the light-dependent consortium or *Rb. sphaeroides* alone resulted in ~3 fold increase of hydrogen production compared to the heterotrophic consortium. Apparently, hydrolysis of starch to hexose was the bottleneck in H_2 photoproduction by the light-dependent consortium. Availability of residual hexose after photofermentation of starch/glucose by the light-dependent consortium or *Rb. sphaeroides* is discussed.

Introduction

Microbial consortia offer a number of benefits compared to individual species, being favorable for survival in natural ecosystems and for application in biotechnology. The basic features are the tolerance for variable growth conditions and the capacity for complete degradation of complex substrates [1]. These features depend mainly on differentiation of functions and communications between members of consortium. The complicated direct metabolite interactions and indirect relationships are well-balanced to maintain the structural stability of the mixed culture [2].

PNSB[1] were reported to be the members of some microbial communities from natural sources. For example, *Rhodobium marinum* was a part of halotolerant community originated from soil treatment sludge, which was able for starch decomposition and H_2 production [3]. Purple bacteria were active in rice soil slurries capable of N_2 fixation [4] and in phototrophic H_2-producing sludge [5,6]. The growth of phototrophic bacteria

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[1] PNSB, purple non-sulfur bacteria; HC, heterotrophic consortium; LDC, light-dependent consortium; Bchl, bacteriochlorophyll; VFA, volatile fatty acids.

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(*Rhodobacter*, *Rhodospirillum*, *Rhodopseudomonas* and *Blastochloris*) was induced from the granules of upflow anaerobic sludge blanket reactor under anaerobic conditions in the light [7,8].

The application of purple bacteria to the hydrogen production systems using wastewater now is the subject of main interest. The following approaches are under consideration: a single-stage photofermentation, a sequential two stage system with separated dark/light fermentation stages and a combined system with simultaneous dark/light fermentations by mixed cultures (co-cultures) in the same vessel [9–13]. Mixed cultures fermentation is considered to be advantageous due to decrease of by-products amount, increase of the hydrogen yield and simplicity in manipulation. Mixed cultures of *Clostridium butyricum* and *Rhodobacter* sp. were successfully used for H_2 production from starch after optimization of growth conditions [14]. Other studies described fermentation of glucose by co-cultures of *Cl. butyricum* and *Rb. sphaeroides* [15], starch wastewater by anaerobic sludge and *Rhodospirillum rubrum* [16], ground wheat starch by *Clostridium beijerinckii* and *Rb. sphaeroides* [17]. Combined dark fermentation and photofermentation of ground/acid hydrolyzed wheat starch was carried out by anaerobic sludge and *Rb. sphaeroides* under various cultivation modes [18–21]. Immobilization of co-cultures appeared to provide some advantages over suspension cultures [22,23]. For mixed culture experiments, different inocula were preliminary grown in dissimilar media, washed and concentrated by centrifugation and then mixed at an optimal ratio. The resulting mixed culture was not used for subsequent inoculation after the experiment.

The development of a new stable mixed culture (consortium) with artificially introduced PNSB had not been reported yet although it would be of practical importance for hydrogen production in a single-stage process. It is believed that similar consortia could be revealed in natural ecosystems. That is why an attempt has been made to develop a long-living starch-degrading consortium including purple bacteria and to compare it with mixed cultures described earlier.

Materials and methods

Bacteria and media

The HC was obtained from silo pit liquid and cultivated as described before [24]. The basic medium with 5 g/l starch was modified by the replacement of peptone with glutamate (10 mM). The recently isolated purple bacterium *Rb. sphaeroides* N7 was grown on Ormerod medium with ammonium sulfate and lactate [25,26]. For hydrogen production experiments ammonium was replaced with glutamate (10 mM), and lactate – with glucose (4 g/l). Mixture of glucose (1 g/l), acetate (10 mM) and butyrate (10 mM) was also used when indicated. Purple bacteria were grown in 16-ml Hungate tubes (8 ml culture) under the air at 28 °C, light intensity – 30 W/m^2.

Development of the LDC

Basic mineral medium for mixed culture contained $FeSO_4$, $MgSO_4$, EDTA and microelements according to Ormerod et al. [26]. It was supplemented with 5 g/l starch. Following solutions with indicated final concentrations were sterilized separately and added before inoculation: yeast extract 0.2 g/l, 100 mM phosphate buffer (pH 7.4) and 10 mM glutamate. Glucose (4 g/l) was used instead of starch when indicated. LDC was grown in illuminated Hungate tubes (16-ml total, 8 ml culture) under the air or Ar gas phase (as specified) until the end of gas production. Experimental conditions were similar to those described above for *Rb. sphaeroides* N7. When required, tubes were incubated in the darkness.

Identification of bacterial species in the HC/LDC

Petri dishes with solidified media (YPS or starch-containing media described in Section 2.2) were inoculated with diluted (1:50) cultures and incubated in the darkness at 30 °C under anaerobic conditions (GasPak™ 100 system, BD) or under the air. Single bacterial colonies from agar surface were picked and spotted in triplicates on the MALDI-TOF sample plate (MSP 96 ground steel targets), covered with 1 μl of the matrix (saturated solution of α-cyano-4-hydroxycinnamic acid in 2.5% trifluoroacetic acid and 50% acetonitrile) and dried in the air. Mass spectra were recorded on Autoflex Speed MALDI-TOF mass spectrometer (Bruker Daltonics, Germany) in a linear positive mode with instrument parameters optimized for the range of 2000 to 20,000 m/z. The appropriate software (Bruker Daltonics, Germany) was used for instrument operation, external calibration, visual spectra inspection and microbial identification. The entire Bruker Taxonomy database containing 4140 library entries was used as the reference database. The software provides a log score used to validate identification at the species level, as recommended by the manufacturer. An MSP-based dendrogram was further constructed with the dendrogram tool embedded in the Biotyper software using default clustering settings and the complete linkage algorithm.

Other methods

Gas production in Hungate tubes (16-ml total, 8 ml culture) was measured every 3–4 days by manometrical approach. The hydrogen production was calculated on the basis of H_2 percentages in gas phase measured by gas chromatography. Bchl concentration was measured spectrophotometrically at 772 nm after extraction with a 7:2 (v/v) acetone:methanol mixture [27]. If grown cultures were heterogeneous, all the measurements were carried out after the thorough mechanical homogenization of cell aggregates. The protein concentration was measured after cells sonication. Metabolites were assayed in the supernatant of culture. The concentrations were determined by gas chromatography for acetate and butyrate [28], lactate-dehydrogenase assay for lactate [29], anthrone assay for residual carbohydrates as hexose [30]. Since acetate and butyrate were the VFAs found in experiments, their sum was given as the total VFAs in Tables and Figures.

Results

Development of the LDC

New LDC was developed by serial passages of inoculum under conditions described in Section 2.2 under Ar or air gas phase.