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Synergistic dark and photo-fermentation continuous system for hydrogen production from molasses by *Clostridium acetobutylicum* ATCC 824 and *Rhodobacter capsulatus* DSM 1710

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ABSTRACT

This study investigated synergistic dark and photo-fermentation using continuous fermentation system (CFS). The system relies on connecting several fermenters from bottom of one to top culture level of the next in a manner that allows for delaying movement of the substrate and thus for its full consumption. While H_2 was collected, CFS allowed for moving liquid byproducts toward the outlet and hence continuous productivity. CFS could be efficiently used for: (1) Continuous dark and photo-fermentation H_2 production by *Clostridium acetobutylicum* and *Rhodobacter capsulatus* producing 5.65 mole H_2 mole^{-1} hexose; (2) Continuous dark-fermentation synergistic H_2, acetone, butanol and ethanol (ABE) production by *C. acetobutylicum* which produced per mole hexose, 2.43 mol H_2 along with 73.08 g ABE (3) Continuous H_2 and methane production by *C. acetobutylicum* and bacterial sludge producing, per mole hexose, 1.64 mol pure H_2 and 2.56 mol CH_4 mixed with 0.37 mol H_2. The hydraulic retention time (HRT) for whole system was short where organic acids produced in dark-fermentation in first fermenter were synergistically utilized for H_2 production by *R. capsulatus* in subsequent fermenters. CFS is suitable for fast-digestible sugars but not lignocelluloses or other hard-digestible organics, requiring prolonged HRT, unless such polymeric organics were hydrolyzed prior to fermentation.

1. Introduction

Various bio-fuels would be of interest in future as alternatives to fossil fuels [1–7] which are close to be exhausted. Bio-hydrogen, bio-methane and liquid bio-fuels such as bio-butanol and bio-ethanol are some of the renewable bio-energies expected to be largely applied in near future [8–16]. These variable forms of bio-energy would be all required in various future applications; however it is currently expensive. Batch fermentation for production of bio-hydrogen, bio-methane and liquid bio-fuels is a tedious process for industrial scale production of these renewable bio-energies. This is mainly due to the need to restart the whole process of fermentation from one round production to the next which require more work-hands and time. Continuous fermentation would be of interest for these bio-fuels production industries.

Hydrogen gas can be produced from organic materials by dark-fermentation using *Clostridium* which produces also organic volatile fatty acids (VFAs) that can either be further metabolized to acetone, butanol and ethanol (ABE) [17–20] or used as organic source of carbon for a second stage mixotrophic H_2 gas production in light by purple non-sulfur bacteria [21–24]. The use of batch fermentation in both cases is a tedious process and requires hard work and time. In this study, a continuous fermentation system (CFS) is described for H_2 gas production by dark and photo-fermentation. The system was investigated for use in continuous dark-fermentation H_2 gas and ABE production and for continuous concomitant H_2 and methane production.

2. Materials and Methods

2.1. Organisms and Culture Conditions

Escherichia coli HD701 mutant bacterium previously constructed [25] from *E. coli* MC4100 (wild-type) was aerobically cultured and maintained on nutrient agar. Prior to H_2 production experiments, it was grown on nutrient broth overnight with shaking (100 rpm) at optimum temperature of 35 °C.

Rodobacter capsulatus DSM 1710 was cultivated in an anaerobic conditions in modified RÄH medium [26,27] containing per liter yeast extract, 0.3 g; sodium acetate, 1 g; K_2HPO_4, 0.5 g; ferric citrate, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; Na_2-succinate, 0.005 g; $CaCl_2.2H_2O$, 0.05 g; 0.4 g; and 1 mL solution of trace elements at pH 6.8. The medium was autoclaved for 15 min at 121 °C. Solution of vitamin B12 (10 mg in 100 mL H_2O) was filter sterilized and 0.4 mL of it was added to the 1 L culture medium before inoculation. The culture medium before inoculation was bubbled with N_2 gas for installing anaerobic condition for

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30 min. After inoculation, the culture was kept in light at 32 °C. A tungsten 100 W lamps were used providing uniform illumination (light intensity: 150–200 W/m^2) at the surface of the glass culture bottles. Inoculation and removing samples from culture was conducted using sterile syringes under nitrogen sparging.

Clostridium acetobutylicum ATCC 824 was grown in a YTG medium at 30 °C under anaerobic conditions [28]. The medium contained (g/L): glucose, 20; yeast extract, 10; tryptone, 30; and L-cysteine, 1. *C. acetobutylicum* spores-formed cells were long term preserved in sterile water at 4 °C as described previously [29]. Prior to H_2 gas production experiments, the spores were activated by re-culturing in growth YTG medium.

2.2. Continuous Dark and Photo-fermentation System for Production of H_2 Gas by C. acetobutylicum and R. capsulatus

H_2 gas production by continuous dark and photo-fermentation system from molasses was conducted at 30 °C. The system composed of 5 connected fermenters where fermenter (I) was inoculated with *E. coli* and *C. acetobutylicum* ATCC 824 for producing H_2 gas by dark-fermentation. The facultative anaerobic bacterium *E. coli* was used for upholding anaerobic conditions. Concomitantly H_2 gas production by photo-fermentation was conducted in photo-fermenters (II), (III), (IV) and (V) that were all inoculated with *R. capsulatus*. The produced gas was collected by CO_2-free H_2 gas collection system as previously described [29]

2.3. Continuous Dark Fermentative Production of H_2 Gas and ABE by C. acetobutylicum ATCC 824 Using CFS

Continuous dark-fermentation for production of H_2 gas and ABE was conducted at 30 °C using CFS. At the start of the experiment only fermenter (I) contained the carbon organic source and the pump was opened for flow of fresh medium after 12 h of the fermentation start time. For dark-fermentation, the five fermenters were inoculated with *E. coli* and *C. acetobutylicum* ATCC 824. The fermentation cultures in all fermenters were maintained at 30 °C with stirring at 100 rpm from the start time of experiments. Hydrogen gas produced was collected by CO_2-free H_2 gas collection system as previously described [29] while acetone, butanol and ethanol (ABE) were collected in the effluent of the system. To investigate the mechanism of the continuous H_2 gas and ABE production system acetate, butyrate, and ABE were measured in all fermenters.

2.4. Continuous Hydrogen and Biogas (Mixed Methane and Hydrogen) Production Using CFS.

The use of CFS for continuous dark fermentative hydrogen and biogas production from molasses was investigated at 30 °C where fermenter (I) was inoculated by *Clostridium acetobutylicum* ATCC 824 while fermenters (II) and up to (V) were inoculated with bacterial sludge. Hydrogen gas produced by fermenter (I) and the biogas (hydrogen and methane) produced by the other fermenters were collected by CO_2-free H_2 or methane gas collection system as previously described [29]. The percent of hydrogen and methane in the collected gas were determined by a gas chromatograph.

2.5. Measurement of Hydrogen and Methane Gases by a Gas Chromatograph.

Gas chromatograph was used for separation of the fermentation produced gases and measurement of H_2 gas. The GC used was of the model (TRACE GC-Ultra, Thermo Scientific). GC used for separation of the fermentation produced gases was equipped with column (stainless steel) filled with molecular sieve 5A (80/100 mesh) 2 m × (1/8) in. × 2.1 mm and HayeSepQ (80/100 mesh) 4 m × (1/8) in. × 2.1 mm. Thermal Conductivity Detector (TCD) was equipped to the GC for detecting the H_2 gas signals. The temperature of the column was started at 50 °C and enhanced at a rate of 30 °C/min to 200 °C. Measurement of methane (CH_4) gas was performed using a gas chromatograph (Thermo Scientific (TRACE GC-Ultra), (Rodano, Italy). GC used was equipped with capillary column (CP-PoraBOND U) fused silica plot (25 m × 0.32 mm, df = 7 mm). Flame Ionization Detector (FID) was equipped to the GC for detecting methane. Nitrogen gas was used at a flow rate of 1 mL/min at 250 °C as the carrier gas. The temperature of the detector was 250 °C while the column and the injector were 37 and 150 °C, respectively. The concentration of acetate and butyrate, ethanol, butanol and acetone were evaluated using GC-FID for samples from each fermenter after 100 h from start of fermentation.

3. Results and Discussion

Batch fermentation has some disadvantages and is not usually suitable for active industrial production of many bio-products as it requires restarting all stages in every round. Thus, for industrial scale, continuous fermentation would be more efficient. In this study, a continuous fermentation system (CFS) was described and its various applications were investigated. The system composed of several fermenters connected in a manner that allows for delaying the substrate movement from a fermenter to the next. Through this way, a full consumption of the substrate occurred for H_2 gas production by dark-fermentation in the first fermenter and photo-fermentation in subsequent fermenters. The VFAs byproducts of dark-fermentation formed by dark fermentation in the first fermenter were synergistically consumed for hydrogen production by photo-fermentation in subsequent fermenters. The system works by moving fermentation medium from a bottom in a fermenter to top culture level of the next one with keeping a slow supply of fresh medium while moving liquid byproducts toward the outlet to avoid feedback inhibition and hence continuous productivity. A pump was used for forcing and controlling the medium flow rate. CFS required five connected fermenters for efficient continuous dark and photo-fermentative H_2 gas production and for continuous dark fermentative H_2 gas and ABE production as well as continuous H_2 and CH_4 production. It is possible to change the number of connected fermenters for full fermentation and substrate consumption.

3.1. Continuous Dark and Photo-fermentation System for H_2 Gas Production by C. acetobutylicum ATCC 824 and R. capsulatus DSM 1710

Dark-fermentation by the acidogenic bacterium *Clostridium* sp. produces H_2 gas and volatile fatty acids [30,31] (e.g., acetic acid, butyric acid) which can be further metabolized where the final fermentation byproducts are acetone, butanol and ethanol (ABE). In another approach, these VFAs can be utilized in light via photo-fermentation with photosynthetic purple non-sulfur bacteria to produce more H_2 gas [32–37]. *R. capsulatus* produces H_2 as a byproduct of nitrogen fixation by nitrogenase that can also work to produce H_2 gas by artificial reaction in absence of molecular nitrogen [32–37]. The two routes require ATP where *R. capsulatus* utilizes VFAs as a carbon organic source for producing such required cellular ATP [32–37]. In this study, a continuous dark and photo-fermentation system (Fig. 1) was used for H_2 gas production at 30 °C from molasses. The continuous system relies on connecting several fermenters where the first one was for dark-fermentation and the subsequent four fermenters were for photo-fermentation. The first fermenter was inoculated with both *E. coli* and *C. acetobutylicum* ATCC 824 where the facultative anaerobic bacterium *E. coli* was used for upholding anaerobic conditions for H_2 gas production by *C. acetobutylicum* (a strictly anaerobic bacterium) [38]. The use of *E. coli* for upholding the anaerobic conditions reduces the cost of the fermentation process instead of using the expensive reducing agents. The fermenters (II) up to (V) were inoculated by *R. capsulatus*. Fermenter (I) contained full medium with carbon organic source in a concentration of 10 g sugars/L. Fermenters (II) to (V) initially contained all medium