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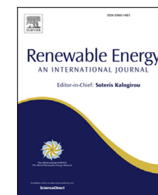

Long term impact of stressing agents on fermentative hydrogen production: Effect on the hydrogenase flux and population diversity

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A B S T R A C T

In this study, the long term effect of different microbial stressing agents on hydrogen (H_2) production was examined using repeated batch cultivations. When compared to thermophilic cultures, higher H_2 yields were observed in mesophilic cultures receiving repeated glucose addition. Methane production was only observed in control mesophilic cultures receiving repeated 5 glucose additions. Lower hydrogenase evolution specific activity was observed in thermophilic cultures (except alkali-treated cultures) compared to mesophilic cultures. For both mesophilic and thermophilic cultures, the hydrogenase uptake specific activity of the untreated control cultures exhibited higher levels of activity than the pretreated cultures. A flux balance analysis (FBA) showed negligible homoacetogenic flux in mesophilic cultures pretreated with linoleic acid (LA) and loading shock (LS) after successive batch cultivations. The homoacetogenic flux accounted for approximately 98% loss in the H_2 yield in untreated mesophilic control cultures. Both homoacetogens (*Eubacterium* sp.) and aceticlastic methanogens (*Methanosaeta* sp. and *Methanosarcina* sp.) were abundant in the control cultures. In comparison, *Clostridium* sp. were dominant in mesophilic stress treated cultures whereas under thermophilic conditions, the dominant microorganisms were *Flavobacterium* sp., *Bacillus* sp., *Thermoanaerobacter* sp., *Bacteroides* sp., *Lactobacillus* sp. and *Thioalkalivibrio* sp.

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1. Introduction

Many global industrial as well as municipal sectors are heavily dependent on the continuous supply of cheap energy. Fossil fuels have been utilized to meet this demand; however, depleting supplies, rising carbon dioxide levels and energy security are major factors driving the search for alternative energy sources [1]. Alternative renewable energy sources in-use and under development includes solar, wind, tidal and biomass. Currently, many studies are focused on developing processes which can utilize agricultural crops and residues to produce biofuels [2]. Cheap renewable feedstocks coupled with economical processes could lead to cheaper supplies of bio-diesel, bio-ethanol and bio-hydrogen (bio-H_2). Among the different biofuels, bio-H_2 is emerging as a promising energy carrier because the reaction byproducts are non-polluting. In addition, with an energy content of 122 kJ g^{-1} and a higher heating value (142 kJ g^{-1}) when compared to other hydrocarbon fuels, bio-H_2 is a promising energy alternative [3,4].

Hydrogen can be produced using thermal, photocatalytic and biological processes. These processes include large-scale thermo catalytic steam reforming, photochemical, thermal and water biophotolysis [5]. Photo-bioprocesses as well as dark fermentation routes under development have utilized lignocellulosic feedstocks [6,7]. Among the biological methods, dark fermentation is the most preferred route because of its high net energy utilization [8] and

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when compared to pure culture, it can use a variety of non-sterile feedstocks. However, using mixed cultures suffers from a severe limitation caused by the presence of H_2 consumers and H_2 producers. Hydrogen is utilized by anaerobic chemolithotrophic bacteria such as methanogens, sulfate reducers and homoacetogens [9,10]. Hence, the successful production of H_2 via dark fermentation depends on the selective inhibition of H_2 consuming bacteria.

Controlling the growth of H_2 consumers can be accomplished by several methods. Strategies employed to selectively enrich spore forming H_2 producing bacteria such as *Clostridia* sp. include inocula pretreatment using acid [11–13], alkali [14], heat [15,16], loading shock [11], 2-bromoethane sulfonate (BES) [17] and long chain fatty acids such as linoleic acid (LA) [18]. Methanogenic archea are mainly inhibited by inoculum pre-treatment [19,20]. However, under conditions favoring methanogenic suppression, H_2 oxidation is able to proceed via homoacetogenesis [21]. Dark fermentation studies have shown that batch studies with a heat treated inoculum, the gas phase H_2 reached intermediate concentrations of 57–72% (head space concentration) from glucose fermentation [22] and after 80 h, the H_2 level was undetectable with acetic acid (HAc) levels increasing after 30 h. The increasing (HAc) levels and decreasing levels of H_2 reported by Oh et al. [22] was likely due to homoacetogens. The data in Table 1 show the impact of inoculum pre-treatment and the effect of homoacetogenesis.

Hydrogen production from single batch pre-treatment of an inoculum have been reported in many studies [14,18,20,22]. Over the long-term, the impact of pretreatment on an inoculum may differ between single batch cultivations because of variable populations in mixed cultures derived from different sources. A proper understanding of the activity of the homoacetogens under conditions mediating methanogenic suppression is crucial and this data could provide insights for developing a H_2 producing inoculum which could be utilized in larger-scale continuous H_2 production. The objectives of this work were to investigate the long term effect of different stress treatments (acid, alkali, heat, BES, LA and loading shock) on H_2 production and to assess changes in the microbial community composition under both mesophilic (37 °C) and thermophilic (55 °C) conditions.

2. Materials and methods

2.1. Chemicals and inoculum source

Experiments were conducted using a mixed anaerobic inoculum from a brewery wastewater treatment facility (Guelph, ON, Canada). The culture was stored at 4 °C before experimentation. The inoculum had a volatile suspended solids (VSS) concentration of approximately 40 g L^{-1}. Two mother reactors were maintained at 37 °C (Reactor A) and 55 °C (Reactor B) with a VSS concentration of 10 g L^{-1}. The reactors were fed glucose (5 g L^{-1}) every 7 days and the gas production was monitored at regular intervals. Before the pretreatment was administered, the VSS concentration of the inoculum was adjusted to 2 g L^{-1}. The volume of gas produced in Reactors A and B was monitored using a tipping bucket gas meter [23]. Heat shock (H) (Moist heat at 100 °C for 30 min), acid (A) (Adjust pH to 3.0 using 3 N HCl for 24 h), alkali (K) (Adjust the pH to 12.0 using 3 M KOH for 24 h), 2-bromoethane sulfonate (BES) (99% purity, Sigma Aldrich, ON) (50 mM 2-bromoethanesulfonic acid for 24 h), linoleic acid (C18:2 (LA) (99% purity, TCI America, OR) (2.0 g L^{-1} for 24 h) and loading shock (LS) (20.0 g L^{-1} glucose 24 h) were used to pre-treat the inoculum.

2.2. Experimental procedures

Long-term studies assessing the impact of different stressing agents on H_2 production was performed using 160 mL batch reactors. Pre-treated inoculum was added to the batch reactors together with glucose and basal media to attain a final working volume of 75 mL. All experimental runs were conducted in triplicate. Culture preparation methods were adapted from Pendyala et al. [18]. Untreated (control) cultures were used as positive controls. The initial pH was adjusted to 5.5 using 3 N HCl/3 M KOH before initiating the experiments. Anaerobic basal media was prepared according to Wiegant and Lettinga [24]. Glucose (5 g L^{-1}) was added to the mesophilic (37 °C) and thermophilic (55 °C) cultures 24 h after applying the pretreatment condition. The batch reactors (160 mL) containing 2000 mg L^{-1} VSS of culture were placed in an shaker incubators (Innova 2100, New Brunswick Scientific, NJ) operating at 200 rpm at 37 °C and 55 °C. At the end of the reaction time after the 1st glucose addition, the pH of cultures in the batch reactor was measured and adjusted to 5.5 ± 0.1. The VSS concentration at the end each glucose feeding (at day 5, 10, 15, 20 and 25) was measured using an optical density (OD) versus VSS concentration curve (data not shown).

Before initiating the second and subsequent glucose feedings, the microbial culture was washed to remove residual VFAs produced from a previous glucose addition. The culture-washing procedure was conducted as follows: 1. Depressurize the batch reactors in an anaerobic glove box (COY Laboratory Products Inc., Grass Lake, MI) by inserting a needle into the rubber septa. 2. Opened the batch reactors and transfer the contents from each triplicate set into 300 mL glass beakers. 3. Gravity settle the solids and decant the supernatant and add fresh basal medium to a final volume of 40 mL. 4. Transfer the culture plus basal medium mixture into a 50 mL centrifuge tube configured with a screw cap. 5. Centrifuge (Beckman Coulter, Inc, USA) the mixture at 3000 rpm for 10 min and decant the liquid. 6. Resuspend the pellet in fresh basal medium after centrifuging and determine the pH and VSS concentration after repeated washing, centrifugation and resuspension.

Table 1
Role of homoacetogenesis in dark fermentative H_2 production.

Inoculum type	pH	Temp. (°C)	Substrate	Type of pre-treatment	H_2 loss due to homoacetogenesis	Reference
MW	6.2	25 ± 1	Glucose	Heat	33% of total H_2 produced	[22]
MW	6.2	24	Glucose	Heat	43% of H_2 produced	[23]
MW	7.5[a]	35	Glucose	Heat	24.3% of total H_2 consumed[b]	[12]
AS	5.5	37	Glucose	Acid, heat, loading shock	56%, 43% and 36% of H_2 produced	[24]
MW	5.3	35	Starch	Heat	57% of H_2 produced	[15]
DDS	5.2	35	Wheat starch	Heat	32% of H_2 produced	[16]

MW = Municipal wastewater.
AS = Activated sludge.
DSS = Digested sewage sludge.
[a] Average pH value was reported.
[b] Average of % electron equivalents from H_2 diverted to HAc was used.

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