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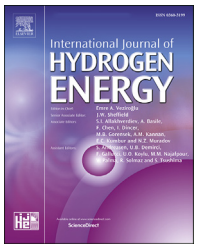

Costless and huge hydrogen yield by manipulation of iron concentrations in the new bacterial strain *Brevibacillus invocatus* SAR grown on algal biomass

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ABSTRACT

There is a growing global demand on bio-hydrogen production (BHP) using costless and wastes material. Herein we demonstrate the possibility to produce high yield of hydrogen using a new bacterial strain grown on acidic hydrolyzed cyanobacterial biomass as a costless carbon feedstock under various iron concentrations. We used *E. coli* DH701 mutant and new strain *Brevibacillus invocatus* SAR isolated from Assiut city soil samples. The mentioned new strain was identified morphologically, biochemically and by molecular analysis using 16S rDNA sequence. Limitation of iron induced BHP in tested cyanobacteria. Iron concentration (0.045 mM) enhanced hydrogenase activity and cumulative hydrogen evolution in the investigated cultures. *B. invocatus* yielded 3.3 mol H_2/mole glucose and 3.8 mol H_2/mole reducing sugar (algal biomass), while the mutant strain yielded 1.78 mol H_2/mole glucose and 3.4 mol H_2/mole reducing sugar (algal biomass). Thus, the use of algal biomass induced higher potency of BHP especially at 0.045 mM iron.

Introduction

Hydrogen (H_2) gas is a promising renewable energy alternative. It is carbon free, renewable, and generates nontoxic byproducts after oxidation or combustion [1]. It has high energy content per unit weight (122 kJ g^{-1}) and thus is an attractive energy source candidate to replace conventional fossil fuels, both from the economic and environmental standpoints. The processes of biological H_2 production are mostly operated at room temperature and pressure. These milder conditions are eco-friendly and energy saving [2,3].

Biohydrogen production (BHP) could be carried out through different biotechniques including using solar energy through various photosynthetic processes, or carrying out an anaerobic fermentation [4]. Dark fermentation has several advantages over photo-fermentation. It does not require light for growth and no oxygen is produced, which act as a strong inhibitor of hydrogenase. Additionally, fermentative BHP also has an additional merit of decomposing organic environmental wastes into more valuable energy resources [5]. Dark fermentation can utilize various organic wastes and wastewaters as a feedstock [5]. Dark fermentation offers relatively higher H2 production rate compared to photo-fermentation Cyanobacteria have been mainly investigated for their capacity to convert captured solar energy to hydrogen. Cyanobacteria possess higher photosynthetic levels and higher cell growth rates compared to algae and higher plants, and can be

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easily grown using basic nutritional requirements such as air ($CO_2 + N_2$), water, and mineral salts with light [6]. Several reports have reviewed freshwater cyanobacteria species capable of BHP [7,8]. On the other hand, dark fermentative bacteria have received a growing attention, mainly with respect to the higher H_2 production in comparison to other biological means [9]. It is promising because it could use numerous readily available waste streams, while at the same time requiring only relatively simple equipment [10].

Different substrates that can be utilized for BHP through dark fermentation can be classified into: (a) First generation feedstocks; comprising carbohydrates such as glucose [11], sucrose [12], starch [13] etc. (b) Second generation feedstocks; constituting lignocellulosic materials originating from non-crop plants or agricultural wastes such as sugarcane bagasse [14], sweet sorghum [15], boiled potato peel [16] etc. (c) Third generation feedstocks; which is comprising microalgae (such as *Spirulina*) [17] and macroalgae (such as *Laminaria japonica*) [18]. Renewable hydrogen production from algal biomass by hydrogen-producing bacteria (HPB) by anaerobic dark fermentation is receiving an increased attention because of its energy saving, eco-friendly, and carbon-neutral characteristics [19,20].

Iron is a critical element in the enzymatic activity responsible for BHP, which can be classified into [NiFe]-hydrogenases, [FeFe]-hydrogenases, and [Fe]-hydrogenase [21–23]. Several studies have reported the effects of iron on the fermentative H_2 production. Some researchers reported enhancement of H_2 production by Fe^{3+} supplementation [24]. Wang and Wan [25] reviewed the effects of Fe^{3+} on anaerobic hydrogen production and reported some inconsistency on the optimal Fe^{3+} concentration. In these studies, different Fe^{3+} concentrations were used. Cui et al. [26], and Lee et al. [27] reported that Fe^{3+} also affects the fermentation pathways.

In the present work, the potential of lab isolate *B. invocatus* SAR was evaluated for BHP exploiting glucose and carbohydrate-rich acidic hydrolyzed algal biomass. Hydrogen production of cyanobacteria was studied. Acidic hydrolyzed algal biomass was used as a biosugar for H_2 production by the novel bacterial strain. The isolate can utilize various substrates comprising simple sugar as well as complex sugar of biological origin thus it can be inferred that the microbe offers dual benefits in terms of managing the bio-residues as well as generating green energy.

Materials and methods

Microorganism and culture media

Oscillatoria angustissmia (*O. angustissmia*) *and Synchocystis* sp. isolated from soil and freshwater Egyptian samples, respectively were used as a model organism in the present study. The microalgae were grown on BG11 modified medium [28], where disodium magnesium salt (0.001 g/L) was used instead of EDTA, ferric ammonium citrate (0.006 g/L) was used instead of iron III citrate as a source of iron, and cobalt nitrate hexahydrate (0.0494 g/L) was used instead of cobalt chloride in the microelement stock solution. This medium was used for growth as well as the preparation of the inoculums.

Cultivation

For standard growth conditions (control) *O. angustissmia* and *Synchocystis* sp. were grown in 500 mL Erlenmeyer flasks containing BG11 medium modified as described by Michel et al. [29]. The pH of the medium was adjusted to pH 7.5 prior to autoclaving. Cultures were incubated at 30 °C with shaking for 6 days. The cultivated flasks were illuminated for 24 h with continuous cool white fluorescent lamp at 400 W (Philips) (48.4 μ mole. m^{-2} s^{-1}).

Experimental design

Prior to the inoculation, the cultures (grown previously in BG11 medium) were centrifuged (6000 rpm, 15 min). The supernatant was decanted, and the cells were washed thrice with the iron-free BG11 medium, then were inoculated in the iron-free or iron containing media; 0.03 mM (control), 0.045 mM or 0.06 mM iron concentration. A multilevel factorial design was used in which the concentrations of iron in the growth medium as the following.

1. Control conditions, where the final concentration of iron was 0.03 mM.
2. Iron-deficient conditions: the inoculum was washed with distilled water, and subsequently was transferred into an iron-free medium and was expressed as (0.00)
3. Prolonged iron deficiency growth conditions: Culture were grown for 7 days in free iron BG11 and then harvested by centrifugation and resuspended in BG11 medium without addition of iron and was expressed as (0.00*).
4. Cultures grown in BG11 medium containing 0.045 mM of iron.
5. Prolonged excess iron growth conditions: culture were grown for 7 days in BG11 medium containing 0.045 mM iron, harvested by centrifugation and resuspended in the same iron concentration and expressed as (0.045 mM*)
6. Cultures grown in BG11 medium with final iron concentration of 0.06 mM.
7. Prolonged excess iron growth conditions: cultures were grown in BG11 medium with 0.06 mM iron concentration, harvest by centrifugation and resuspended in BG11 medium with the same iron concentration, and was expressed as (0.06 mM*)

Isolation and identification of bacterial culture

The bacterial strain (*B. invocatus* SAR) was isolated from soil, Assiut city on nutrient broth at 37 °C for overnight. A loopful of the culture was streaked on nutrient agar and incubated at 37 °C for 18 h. The bacterial isolate was morphologically and biochemically characterized according to Bergy's manual of systemic bacteriology [30]. Also, this bacterial isolate was further identified by 16S rRNA sequencing. The culture was maintained on a nutrient agar plates/slants at 4 °C and as glycerol stocks 40% at −70 °C.

Molecular typing

Molecular characterization using 16S rRNA gene

Some modifications have been displayed to The Genomic DNA Purification Wizard Kit (Promega, USA) as follows: Prior to