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Original Research Article

Identification and enhanced hydrogen evolution in two alginate-immobilized strains of *Brevundimonas diminuta* isolated from sludge and waterlogged soil

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ABSTRACT

As hydrogen gas is a clean energy carrier, finding out strains of higher hydrogen evolving capacity than those recorded in the literature is the target of this work. Two bacteria were isolated from sludge and water logged soil samples at Arab Elmadabegh (Assiut) and Sohag (Upper Egypt). Phenotypic, genotypic as well as electron microscopic characterization revealed that they are two strains of *Brevundimonas diminuta* (B1 and B2). They both are motile short rods, Gram negative, acid and hydrogen producers. However, growth (O.D. 660 nm) in control cultures of B1 was relatively higher than B2, and protein contents were markedly the opposite while hydrogen yield of B1 was almost double that of B2. The hydrogen yield of control cultures occurred at the first 72 h giving 570 and 300 ml/culture of B1 and B2, respectively. Alginate immobilization supplemented with $CaCO_3$ greatly elevated the efficacy of hydrogen production to 1200 ml/culture for B1 and 1300 ml for B2. Variation in the enhanced level of hydrogen evolution by $CaCO_3$ may indicate variation in the amount of acid produced. The hydrogen produced is exclusively a hydrogenase enzyme activity since both strains did not exhibit any nitrogenase activity. Hydrogenases (Hox and Hup) of the two strains exhibited their maximum activity rates at 72 h that is similar to their growth and hydrogen yield.

1. Introduction

In addition to the highly hydrogen producing PNS (purple non-sulfur bacteria), some heterotrophic (N_2-fixing or non-fixing) bacteria are also active hydrogen producers. The production of hydrogen from *Bacillus polymyxa* and other heterotrophic bacteria was cited since a long time (Grau and Wilson, 1962). In addition, other non-N_2-fixers were reported to produce H_2, *Clostridium*, *Escherichia coli*, etc. (Penfold et al., 2003). Taguchi et al. (1996) reported continuous H_2 production by *Clostridium* sp. strain No. 2. In addition, Kotay and Das (2002) reported H_2 production with *Bacillus coagulans* IIT-BT S1 isolated from anaerobic sewage sludge. Elsamadony et al. (2015) reported hydrogen production from organic fraction of municipal solid waste (OFMSW) via dry anaerobic digestion. Kalia et al. (1994) described the fermentation of biowaste to hydrogen by *Bacillus licheniformis*. Mixed

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culture of *Clostridium butyricum* and *Enterobacter aerogenes* can produce H_2 from starch (Haruhiko et al., 1998). Kalia et al. (1994) described the fermentation of biowaste to hydrogen by *B. licheniformis*. Mixed culture of *C. butyricum* and *E. aerogenes* can produce H_2 from starch (Haruhiko et al., 1998). In addition, Danial and Abdel-Basset (2015) reported hydrogen production from orange peel with some purple non sulfur bacteria. *E. coli* HD701 was able to evolve significant amount of hydrogen using some waste fermentation products (Abdel-Basset, unpublished data). Immobilization has been repeatedly recorded to enable higher rates of cellular activities at the expense of growth, which is usually slowed down. Several inoculums formulation has been proposed including alginate and agar immobilization inoculants (Bashan et al., 2002; El-Komy, 2001). These carriers permit entrapment of living cells, protecting the organisms against stresses. In addition, microbial immobilization promotes a slow release of bacteria into soil (Bashan, 1986; Shaban and El-Komy, 2000; El-Katatny et al., 2003). Immobilized-cell systems are also alternative to suspended-cell systems in continuous operations for enhanced biohydrogen production because they are capable of maintaining higher biomass concentrations and could operate at higher dilution rates without biomass washout (Show et al., 2008). Biomass immobilization can would be achieved through forming granules, biofilm, or gel-entrapped bioparticles, which have been employed in different reactor systems (Zhang et al., 2008).

Calcium, on the other side, is an important factor in maintaining membrane integrity and thus regulation of ion transport. It has also been shown that Ca^{2+} is essential for K^+/Na^+ selectivity and membrane integrity (Epstein, 1961; Läuchli and Epstein, 1970; Hanson, 1984). Ca^{2+} also interferes with energy metabolism at least by forming insoluble precipitate of phosphate and with carbon nutrition by forming insoluble precipitate of carbonate. Danial (2008) found that Ca^{2+} enabled some *Bacillus* Spp. of tolerating high salinity concentrations and normalized their metabolism. Within the context of this work, $CaCO_3$ has being used to neutralize the medium for bacteria as some strains acidify the medium by secreting formic acid to the medium (Sakano et al., 1997).

The objective of this study was to search for new hydrogen producing bacteria, may be of higher capacities to evolve hydrogen than the recorded ones. An additional approach was to intensify hydrogen production of the two newly isolated bacterial strains by immobilization and neutralization of the acids produced. The strains have been identified and characterized according to their morphological, biochemical, phenotypical, and genetic traits, they exhibited.

2. Materials and methods

2.1. Soil samples, isolation and identification (characterization) of the heterotrophic bacteria

Samples from sewage and waterlogged soils were collected from Assiut University and some other localities near-by Assiut and Sohag city. Purification of isolates was carried out on NA (nutrient agar) media. Bacteria were grown for 5–6 days at a temperature of 30 °C, pH 6.8–7 under aerobic conditions.

Preliminary identification was carried out according to the methods described in Bergey's Manual (Sneath et al., 1984; Brenner et al., 2005). Isolates were phenotypically examined (Gram stain, motility, morphological and biochemical characteristics).

2.2. Biochemical tests

For characterization and identification of the isolates, keys of Bergey's Manual were followed. Diagnostic media of general use was prepared as recommended by Harrigan and Mecance (1966), specific media for the identification of isolates was also prepared, and motility was tested by SIM (sulfur indole motility test) medium (Difco. 1998, Difco manual, 11th ed. Difco Laboratories, Detroit, MI.), confirmed by microscopic examination. The tests applied for identification (characterization) were catalase (Wittenberg, 1964), differentiation of oxidation and fermentation of glucose (Hugh and Leifson, 1953), gelatin hydrolysis (Smith et al., 1952), starch hydrolysis (Harrigan and McCance, 1966), nitrate reduction (Breed et al., 1957), acid production from carbohydrates and the utilization of different carbon sources (Gordan and Mihm, 1957).

2.3. Genotypic identification

Attempts for identification of our isolates were done on the bases of 16S rRNA base sequence analysis after extraction of total genomic DNA (Wu et al., 2000) followed by PCR amplification of the 16S rRNA gene. The PCR product was sequenced using DNA sequencing Services in Sigma Company in South Korea.

2.4. Analytical methods

Turbidity (O.D.): For fast assessment of growth, O.D. 660 nm was followed each 24 h, over a period of 4 days spectrophotometrically (Thermo Scientific spectrophotometer, Germany). All the experiments presented in this work were started by a constant inoculum giving a final absorbance of 0.2 A.

Determination of soluble protein contents: Water-soluble (1 h boiling of bacterial cells) protein concentrations were estimated according to the method of Lowry et al. (1951).

Estimation of H_2 produced: Hydrogen evolved was collected and estimated in an inverted graduated glass cylinder over water. Hydrogen was, then confirmed by TCD-GC (Thermal Conductivity Detector Gas Chromatography, Thermo Scientific, Germany).

Assessment of hydrogenase activity (Hup and Hox): Uptake activity of the bidirectional hydrogenase (Hup) has been assayed in mixture containing methylene blue flushed with hydrogen as it was conducted by Yu et al. (1969) and Colbeau et al. (1980). The evolution activity of the bidirectional hydrogenase (Hox) was applied similar to that used by Schultz et al. (2004) and Kim et al. (2008a,b) using dithionite-reduced methyl viologen flushed with nitrogen.