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Short Communication

Enhancement in hydrogen production by co-cultures of *Bacillus* and *Enterobacter*

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ABSTRACT

Defined co-cultures of hydrogen (H_2) producers belonging to *Citrobacter*, *Enterobacter*, *Klebsiella* and *Bacillus* were used for enhancing the efficiency of biological H_2 production. Out of 11 co-cultures consisting of 2–4 strains, two co-cultures composed of *Bacillus cereus* EGU43, *Enterobacter cloacae* HPC123, and *Klebsiella* sp. HPC793 resulted in H_2 yield up to 3.0 mol mol^{-1} of glucose. Up-scaling of the reactor by 16-fold resulted in a corresponding increase in H_2 production with an actual evolution of 7.44 L of H_2. It constituted 58.2% of the total biogas. Continuous culture evolution of H_2 by co-cultures (*B. cereus* EGU43 and *E. cloacae* HPC123) immobilized on ligno-cellulosic materials resulted in 6.4-fold improvement in H_2 yield compared to free floating bacteria. This synergistic influence of *B. cereus* and *E. cloacae* can offer a better strategy for H_2 production than undefined or mixed cultures.

Introduction

Fossil fuels are used extensively worldwide to conform to the exponentially increasing energy needs. Continued reliance on fossil fuels as primary energy resources is becoming unsustainable due to limited world fuel reserves. Vigorous research initiatives are directed at developing alternative renewable energy resources. Among the diverse available alternative sources, hydrogen (H_2) has been distinguished as an attractive energy carrier due its high energy (122 kJ/g) content and environmentally friendly nature [1–3]. Among

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the different methods being explored, biological H_2 production (BHP) has gained significant importance due to its production under ambient physiological conditions and identification of many H_2 producers [1,4–9]. BHP has been reported under dark-fermentation by pure strains of *Bacillus*, *Citrobacter, Clostridium, Enterobacter* and *Klebsiella* or under the photo-fermentation by *Rhodobacter*, *Rhodopseudomonas*, *Rhodospirillum*, etc. [10–15]. Each microbial strain has limitation towards H_2 production under different fermentative conditions. In most cases, lower H_2 yields were observed than the theoretically achievable yield of 4 mol mol^{-1} of hexose [3,13,16]. Attempts are still being made to search for the potential candidates for the H_2 production, which – i) can adapt to a broad range of physiological conditions, including nutrient limitation, ii) can utilize diverse substrates (including biowastes), and iii) have an ability to compete with other microbes present in unsterilized feed on a large scale [1–3]. Altogether these characteristics are important for the economic system of the BHP process. Various mixed cultures have been applied for improving in H_2 production, but yields continue to be quite low, in the range of 2.5–3.0 mol mol^{-1} [13,17–19]. In comparison to pure cultures, co-cultures could be more suitable for synergistic effects on H_2 yields. Research is required for complementing the abilities of different strains to develop robust co-cultures to achieve the goal of the sustainable BHP system [20]. The most readily proposed mechanism is genetic engineering. However, we can expand the range of biosynthetic and metabolic pathways by using co-cultures of different organisms. H_2 evolution in microbes operates through the involvement of enzymes: nitrogenase, and hydrogenases [5]. Fermentative organisms such as *Enterobacter* catalyze pyruvate to H_2 via formate and protons, with the help of enzyme formate dehydrogenase and hydrogenase. Nitrogenase uses Mg^{2+}, ATP and low-potential electrons derived from ferredoxin or flavodoxin and reduces a number of substrates. However, in the absence of these substrates it reduces protons to H_2. In *Bacillus*, H_2 production perhaps occurs through formate dehydrogenase than via hydrogenase [2,5]. More recent works have revealed the presence of hydrogenase genes in *Bacillus* spp [2]. In the present study, we can expect a complementation of two different H_2 production pathways to be operative. A major limitation of BHP, is the sensitivity of the H_2 producing enzymes to oxygen (O_2) [1]. In fact, syntrophic associations involving different species of *Bacillus* and *Clostridium*, *Bacillus* proved to be an effective consumer of oxygen and consequently in improving fermentative H_2 production process [21,22]. Facultative anaerobe, *Enterobacter aerogenes* helped *Clostridium* to produce H_2 effectively by consuming O_2 present in the reactor [1]. In other studies, combinations of *Bacillus coagulans*, *Citrobacter* and *Enterobacter cloacae* lead to enhanced H_2 yields because of the effective hydrolytic enzyme activities of *E. cloacae* and *B. coagulans* [11].

In the present study, we have evaluated the complementarities of the H_2 production potential of *Bacillus*, *Citrobacter*, *Enterobacter* and *Klebsiella* strains isolated from various habitats. A significant enhancement of H_2 yields was observed with co-cultures. High H_2 yields were quite stable under batch culture as well as on up-scaling. Continuous culture production of H_2 using immobilized co-cultures on ligno-cellulosic biowaste materials has been also demonstrated. The metabolic activities of these strains may be complemented to use biowaste materials as feed to increase the efficiency of the H_2 production process.

Material and methods

Organisms and growth conditions

Bacterial strains used in this study were isolated previously in our laboratories (Table A1, http://www.ncbi.nlm.nih.gov/). All the strains were maintained on nutrient agar at pH 7.0, containing salt (0.5% NaCl) and grown on HiMedia nutrient broth at 37 °C at 200 rpm for 16–20 h [13,15].

Batch culture hydrogen production

By pure cultures

Initial screening of bacteria belonging to genera: *Citrobacter*, *Enterobacter* and *Klebsiella* for H_2 production was done under batch-culture conditions. 250 mL of minimal medium (M-9) supplemented with 1% (w v^{-1}) glucose in 300 mL BOD bottles were inoculated with different strains at the rate of 10 μg cell protein mL^{-1}, at pH 7.0, and incubated at 37 °C. The evolved gases were collected by water displacement method and residual glucose was measured by DNSA method [13,15].

Four strains with the highest H_2 producing abilities in their respective genus were tested further at different glucose concentrations (0.5–2.0% w v^{-1}) on: i) M-9, ii) GM-2 (Yeast extract – 1.0 g L^{-1}, K_2HPO_4 – 1.0 g L^{-1} and $MgSO_4.7H_2O$ – 0.5 g L^{-1}), and iii) YMG (Yeast extract 10.0 g L^{-1} and Malt extract 10.0 g L^{-1}) [13]. The values are based on three sets of observations.

By co-cultures

Citrobacter sp. HPC1212 (AY948270), *E. cloacae* HPC123 (DQ129709), *Klebsiella* sp. HPC793 (AY838383) and *Bacillus cereus* EGU43 were used for preparing 11 co-cultures: 2MC1–2MC6 (2 strains each), 3MC1–3MC4 (3 strains each) and 4MC1 (4 strains) (Table 2). These co-cultures were prepared by mixing bacterial cultures in equal proportions, amounting to a final cell protein concentration of 10 μg mL^{-1} feed. Their H_2 producing abilities were tested on: i) M-9, ii) GM-2, and iii) YMG media, containing 0.5% glucose (w v^{-1}).

Up-scaling of hydrogen production by co-cultures

Up-scaling of H_2 producing co-cultures (2MC2 and 2MC6) were performed under batch culture using 0.75, 1.5 and 4 L feed (YMG) containing 0.5% glucose in reactors of 1, 2 and 5 L capacities, respectively.

Continuous culture hydrogen production using immobilized co-culture

Dried ligno-cellulosic materials – Banana leaves (BL), Coconut coir (CC), Groundnut shells (GS) or Pea shells (PS) were packed in Polyvinylchloride (PVC) tube (length: 3 cm and diameter: 2 cm) and tied with a 10 cm^2 nylon net to develop a cartridge for immobilizing bacteria, as described in previous study [13].