Feasibility of biohydrogen production from sewage sludge using defined microbial consortium

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ABSTRACT:

Biological hydrogen production potential of a defined microbial consortium consisting of three facultative anerobes, *Enterobacter cloacae* IIT-BT 08, *Citrobacter freundii* IIT-BT L139 and *Bacillus coagulans* IIT-BT S1 was studied. In this investigation their individual and combinatorial H_2 production capabilities have been studied on defined media and pretreated sewage sludge. Defined medium, MYG (1% w/v Malt extract, 0.4% w/v yeast extract and 1% w/v glucose) with glucose as limiting substrate has been found to be most suitable for hydrogen production. Individually *E. cloacae* clearly gave higher yield (276 ml H_2/ g COD reduced) using defined medium than the other two strains. There was no considerable difference in maximal yield of hydrogen from individual and combinatorial (1:1:1 consortium) modes suggesting that *E. cloacae* dominated in the consortia on defined medium. Contradictorily, *B. coagulans* gave better biohydrogen yield (37.16 ml H_2/ g COD consumed) than the other two strains when activated sewage sludge was used as substrate. The pretreatment of sludge included sterilization, (15% v/v) dilution and supplementation with 0.5%w/v glucose which was found to be essential to screen out the hydrogen consuming bacteria and ameliorate the hydrogenation. Considering (1:1:1) consortium as inoculum, interestingly yield of hydrogen was recorded to increase to 41.23 ml H_2/ g COD reduced inferring that in consortium, the substrate utilization was significantly higher. The hydrogen yield from pretreated sludge obtained in this study (35.54 ml H_2/ g sludge) has been found to be distinctively higher than the earlier reports (8.1 – 16.9 ml H_2 / g sludge). However it was lower compared to the yield obtained from co-digestion of (83:17) food waste and sewage sludge (122 ml H_2/ g carbohydrate COD). Employing formulated microbial consortia for biohydrogen production from sewage sludge was an attempt to augment the hydrogen yield from sludge.

KEYWORDS: *H_2 yield, microbial hydrogen production, microbial consortium, process optimization, sewage sludge.*

Introduction

Hydrogen is now universally accepted as an environmentally safe, renewable energy resource and an ideal alternative to fossil fuels that doesn't contribute to the greenhouse. Since, production of hydrogen from fossil fuels and by other conventional means is concurrent with CO_2 generation, biological production is considered as an efficient alternative. Furthermore these techniques are well suited for decentralized energy production in small-scale installations in locations where biomass or wastes are available, thus avoiding energy expenditure and costs for transport.

Through biological hydrogen production process microorganisms can recover and concentrate the energy from high water content organic resources such as waste effluents and sludge in a usable form. So, biohydrogen production in a sense is an entropy reducing process, which could not be realized by mechanical or chemical systems [1,2]. Furthermore, renewable biomass can be used as substrates for biological H_2 production facilitating both bioremediation and energy recovery.

Nature in the form of microorganisms has been using hydrogen as a primary fuel source for billions of years, and has solved the problem of converting hydrogen to electricity by means of the biocatalyst, *hydrogenase*. Although they catalyze a deceptively simple reaction, they have proved to be some of the most complex and ingenious bioinorganic structures known. Microorganisms generate hydrogen for two principle reasons, first to dispose of excess reducing equivalents or as a byproduct in nitrogen fixation. Microbial H_2 production is an attractive process for supplying a significant share of the H_2 required for the near future. Galore microbial species, belonging to the genera *Enterobacter*, *Citrobacter, Bacillus*, and *Clostridium* are reported to produce hydrogen through dark fermentation [1]. Apart from pure cultures, various mixed micro-flora and co-cultures have also been explored for hydrogen production from carbohydrates [1-3]. Nevertheless, the quest for ideal microbe(s) for microbial H_2 production has thrust the researchers to screen various sources.

On the contrast, several substrates have been experimented for the maximization of biological hydrogen production. The feasibility of fermentative H_2 production from organic wastes or wastewaters has been widely demonstrated by various laboratories [4-9]. Hypothetically, development of technology for conversion of waste to molecular hydrogen has the potential to address the several economic and environmental issues. Sewage sludge is an important renewable biomass energy source, which unlike others can be more harmful to the environment if not utilized or properly disposed. The high nutrient content makes it an ideal consideration as fermentative substrate especially in biological hydrogen production. The

prime advantage is expensive management and disposal of sewage sludge is surmounted and the amount otherwise required for disposal can be converted into a credit against the cost of H_2 production.

Sewage sludge typically consists of 41% protein, 25% lipid, 14% carbohydrate, and 20% unknown components on the basis of COD [10]. But, mostly present in non-utilizable form; hence pretreatment becomes essential to render them suitable for H_2 fermentation [5]. Another credible option is co-digestion with other carbohydrate rich biomass like food waste [6] or molasses. However, there remains considerable effort yet to be put in this aspect of study. Most studies on biological H_2 production from sewage sludge have exploited enriched, undefined microbial consortia or pure microbial strains.

The present study on one hand is inspired from previous studies to employ pre-treated sewage sludge as the primary substrate for microbial hydrogen production, compare the H_2 yield with that from pure substrate and from another standpoint investigate the potential of a defined microbial consortium for the purpose. This endeavor would to a great extent solve the problem of sewage sludge disposal and concurrently attend several limitations of biological hydrogen production.

Materials and methods

Bacterial growth conditions and culture maintenance

All hydrogen producing bacteria used in the study were cultured on growth media, nutrient broth (Himedia laboratories), consisting of peptic digests of animal tissue 5.0 g/l, sodium chloride 5.0 g/l, beef extract 1.50 g/l, yeast extracts 1.50 g/l. Initial pH of the media was adjusted to 6.0 prior to sterilization. The liquid cultures for inoculum were uniformly grown at 37°C in an incubator-shaker at 250 rpm, for 12h (expect in the study concerning the effect of inoculum age).

16S rDNA sequencing, phylogenetic analysis and microbial characterization

Genomic DNA of the bacteria was isolated using the method described by Marmur 1961. The primers designed from 16S rDNA conserved gene sequences of γ-Proteobacteria were employed to amplify the ~1.5 kb long fragment [11]. The sequencing was done at Sequencing facility, MWG Biotech, Germany. Clone Manager suite 5.0, SE Central software was used in the sequence analysis, alignment and to obtain the phylogenetic tree.

Hydrogen production in batch fermentors

Experiments were carried out in jacketed glass reactor (of working volume 500 ml) placed on a magnetic stirring platform using the free suspended cells of the isolates. Production medium, MYG (containing 1% w/v malt extract, 0.4% w/v yeast extract, and varied concentrations of glucose) was used for fermentative studies. All the media and glassware were autoclaved for 15 min at 121°C and 1.05 kg/cm^2

steam pressure. Anaerobicity was maintained by flushing with argon for fixed time after the inoculation. The effluent from the reactor was collected timely for estimation of glucose utilization or COD reduction. The gas mixture consisting of CO_2, H_2 and other gases was allowed to pass through 40 %w/v KOH solution for selective absorption of CO_2. The filtered gas was collected in a graduated water displacement system containing saline solution at ambient pressure and room temperature.

Analytical methods

i) Hydrogen

The composition of the gas (in the collector) was analyzed by a Gas chromatograph (Perkin-Elmer) with TCD using Porapak-Q column using N_2 as carrier gas at 5.62 kg/cm^2 pressure and 20 ml/min flow rate. Injector, oven and detector temperatures were set at 150°C, 80°C and 200°C respectively. The chromatogram was developed and analyzed using software, Turbochrome Navigator (version 4.1) Perkin-Elmer Coorp.

ii) Physico-chemical characteristics

Physical parameters viz, T.S, T.S.S., T.V.S., T.D.S., B.O.D etc. were characterized using protocols mentioned in Standard Methods, [12]. C.O.D estimation was performed using $K_2Cr_2O_7$ open-reflux method [13] and T.O.C using Rapid Titrimeric method [12].

iii) Glucose concentration

Reducing sugar content of the medium was estimated spectrophotometrically (UV-VIS Spectrophotometer Perkin Elmer, λ = 630 nm) using Anthrone reagent [14].

iv) VFA and alcohols

Qualitative detection of VFA and alcohols in the distillate of spent media was carried out with GC using FID Detector, in capillary column at preset injector, oven and detector temperatures, 250°C, 170°C and 280°C respectively.

Results and discussion

Phylogeny of the hydrogen-producing strains

Investigators have explored sewage sludge for microbial strains having superior H_2 production potential [7-8,15-18]. In the present investigation, one bacterium with superior hydrogen production ability, viz. *Bacillus coagulans* IIT-BT S1 that was screened in earlier studies was employed along with two other reported strains *Enterobacter cloacae* IIT-BT 08 and *Citrobacter freundii* IIT-BT L139 in individual and in

consortium. At the primary level it was found necessary to understand the phylogenetic relationship of these bacteria to understand their metabolism and to exploit them in an efficient manner.

The ~1.5 kb 16S rRNA gene amplicons were PCR amplified from the genomic DNA of the organisms using the primers designed. The alignment of 16S rRNA fragments with the established sequences from NCBI database facilitated to deduce the phylogenetic tree (Figure 1). The *Enterobacter* and *Citrobacter* strains showed phylogenetic relatedness with other bacteria in γ-Proteobacteria while *Bacillus* strain was more related phylogenetically to *Clostriudium.* The members of these groups have been known from earlier works for their potential hydrogen yields. Interestingly, these bacteria are metabolically versatile, grow in minimum mineral salts, utilize a wide range of carbon compounds as carbon and energy source. Several bacteria (including the above three) have been expansively studied for their individual hydrogen production abilities over pure and complex substrates in earlier reported works. The three strains uniquely standout in their superior hydrogen production ability and hence the endeavor was to study their abilities in conjunction.

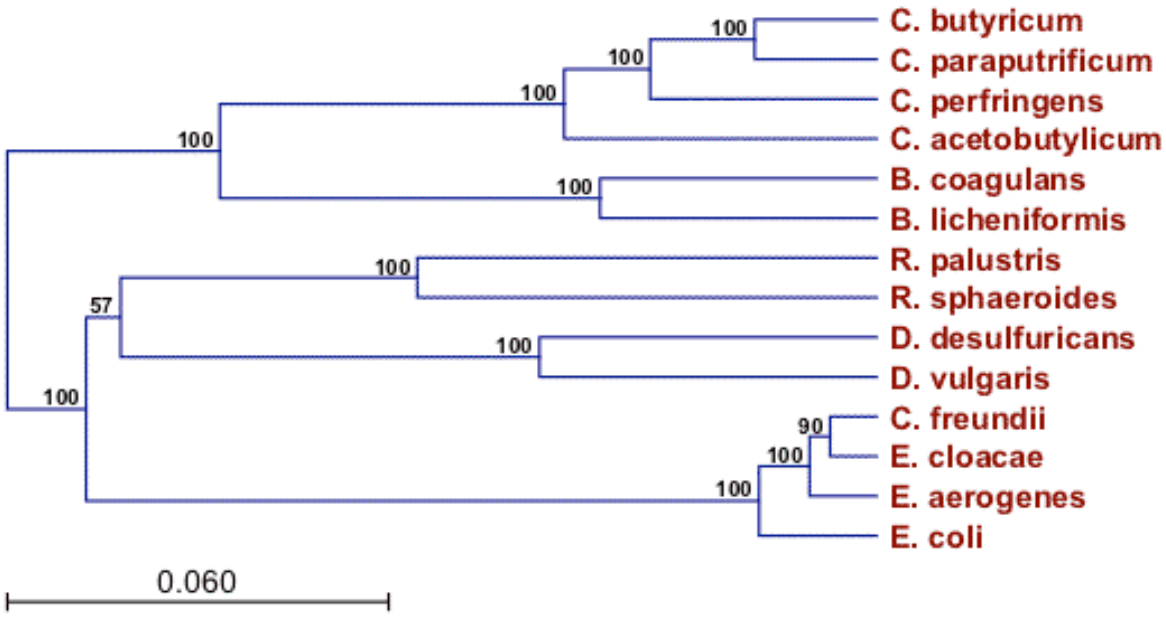

Figure 1. Phylogenetic tree depicting the lineage among the established potential H_2 producing strains

Hydrogen fermentation

This has been a unique attempt to study a defined microbial consortium consisting of three bacteria derived from different sources. So, primarily their hydrogen production ability was tested on defined medium, MYG (1% w/v Malt extract, 0.4% w/v yeast extract and 1%w/v glucose) with glucose as limiting substrate, which has been found in earlier studies to be most suitable for hydrogen production. Optimal conditions of pH, temperature, inoculum age and inoculum volume 6, 37^{o}C, 12 h and 10% v/v respectively were considered as maintained in previous reports.

Individually, strain of *E. cloacae* clearly gave higher yield (276 ml H_2/ g COD reduced) using defined medium than the other two strains (Figure 2). There was no considerable difference in maximal yield of hydrogen from individual and combinatorial (1:1:1 consortium) modes suggesting that *E. cloacae* dominated in the consortium when defined medium was used. Another interesting fact observed was the reduction in COD was maximal in case of *E. cloacae*, supporting its superior ability to convert glucose to H_2.

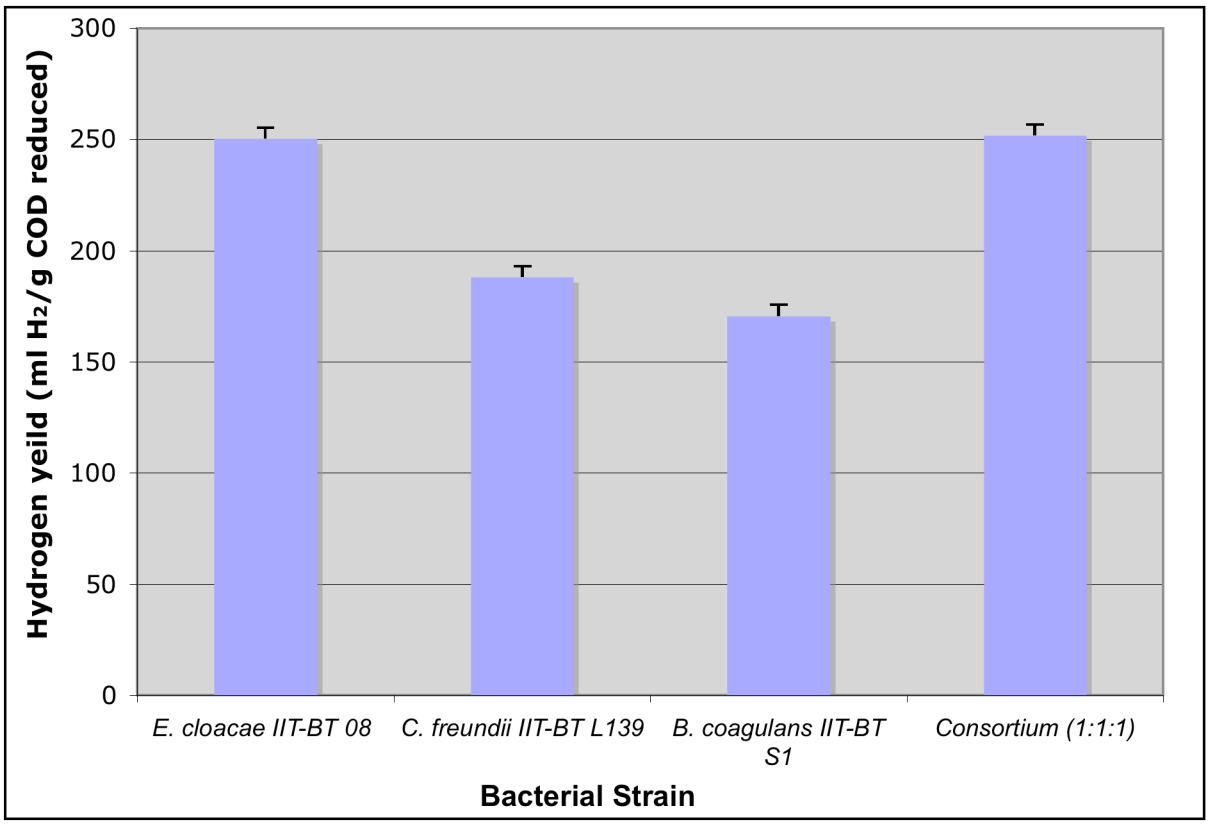

Figure 2. Comparative hydrogen yield using defined medium, MYG.

Contradictorily, strain of *B. coagulans* gave better biohydrogen yield (37.16 ml H_2/ g COD reduced) than the other two strains when activated sewage sludge was used as substrate (Figure 3). However, the yield from sludge was far below than that from defined medium, MYG. Considering (1:1:1) consortium as inoculum, interestingly yield of hydrogen was recorded to increase to 41.23 ml H_2/ g COD reduced. This inferred that in consortium, the substrate utilization is significantly higher. Further to support this, it was found that COD reduction was maximal when consortium was used. Especially since sludge is a complex substrate the total nutrients solubilized by the consortia should be higher than that by individual bacteria.

The hydrogen yield from pretreated sludge obtained in this study (35.54 ml H_2/ g sludge) has been found to be distinctively higher than the earlier reports (8.1 – 16.9 ml H_2/ g sludge) [4,5]. It was lower compared to the yield obtained from co-digestion of (83:17) food waste and sewage sludge (122 ml H_2/ g

carbohydrate COD) [9]. However, it must be noted that in the above study food waste was the major component not sewage sludge.

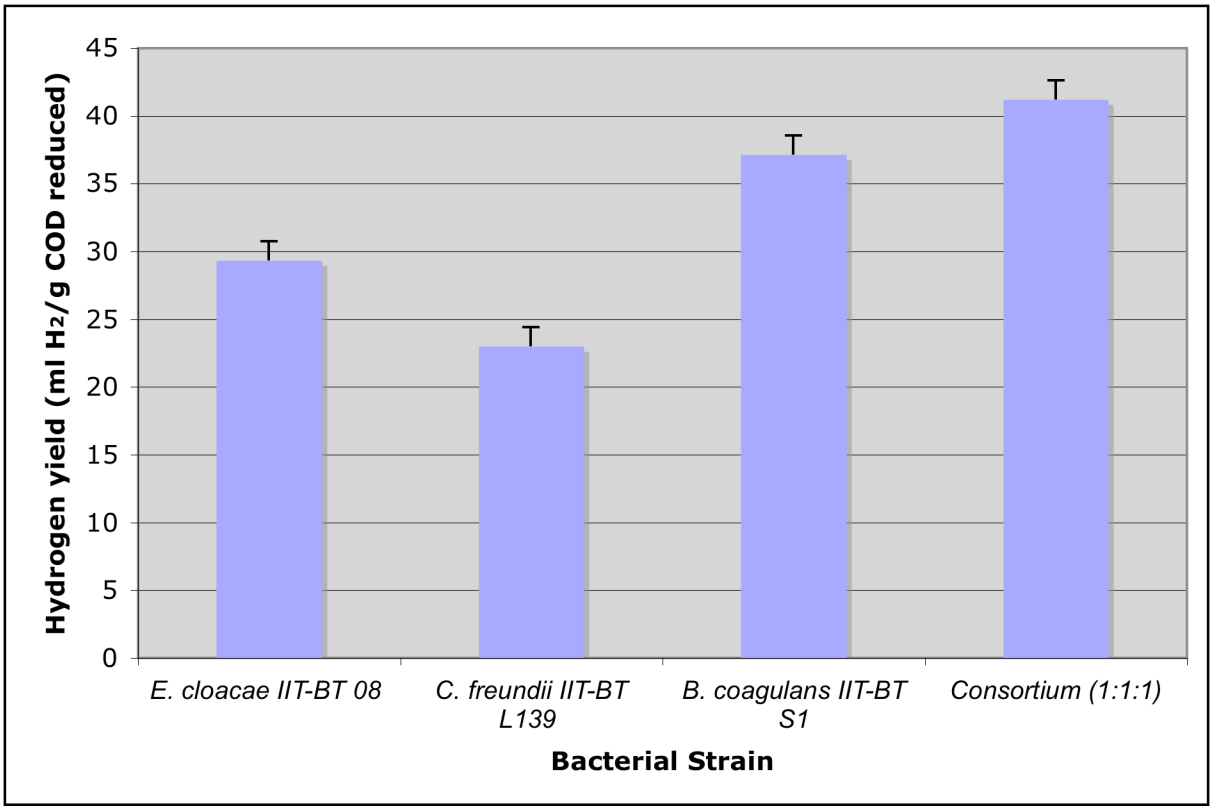

Figure 3. Comparative hydrogen yield using pretreated sewage sludge.

The unit denotation by the authors to depict the hydrogen yield from sludge and biological hydrogen production in general has to be more consistent for better comparison.

Conclusions

Biohydrogen production from renewable substrates is a promising element in the sustainable hydrogen economy. Very little work has been done on biohydrogen production from renewable substrates using defined or complex microbial consortia. Though raw sewage sludge is abundant in several nutrients, the low H_2 yield from it suggests the need for nutrient and seed formulation so as to augment its exploitation as substrate for H_2 production. Different pretreatment techniques coupled with optimal dilution and supplementation is an attempt in this direction. Co-digestion, whole cell immobilization and process optimization should prospectively help in attaining the critical yield value that can upgrade the process for commercial exploitation.

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