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Abstract

Biological production of hydrogen has been investigated over the past 30 years with the ultimate goal of providing a clean, carbon-neutral fuel. However, based on an extensive literature search and the recommendations of several recent DOE- and DOD-sponsored expert review panels it is obvious that an important element of this research has been largely overlooked - the physiology and diversity of naturally occurring, H_2-producing bacteria. The main objective of this project was to develop a technique to extensively screen nitrogen fixing bacteria isolated from unique environments suspected of H_2 production. Those showing H2-producing activity were tested on latex based mats, which could provide active centers of fuel cells. Specific objectives of the project were to establish a biofuels laboratory at the Aiken County Center for Hydrogen Research, where the following activities were persued.1) Develop a semi-automated apparatus to screen hundreds of bacteria in a short time; 2) Identify bacteria capable of producing hydrogen at rates sufficiently high to power a fuel cell. 3) Embed specific bacteria with high hydrogen production potentials into latex mats that can be incorporated in fuel cells applicable to a variety of industrial settings.

During this project we developed screening techniques which include colorimetric and gas chromatographic assays for hydrogen production by bacterial isolates. Isolates were characterized both metabolically and genetically and preserved for future use. Isolates found to produce significant amounts of hydrogen were screened for activity under various environments. Potential isolates were then embedded in latex coatings and assayed for hydrogen production under different environmental conditions

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Introduction

As the worldwide demand for energy approaches the readily available supply of fossil fuels, alternative energy sources are becoming a major focus of research and development. One of the most attractive alternative fuel sources is biohydrogen produced from phototrophic microorganisms (1, 8, 16, 24). This process consumes CO_2, produces H_2 and O_2, and is driven by energy from sunlight and electrons from water, thus it requires few resources and is carbon neutral (31). One of the inherent problems with using these organisms for biohydrogen production is that as their biomass increases in liquid culture, their rate of growth and hydrogen production becomes limited due to self-shading (3, 6, 26). This is especially important to overcome for large-scale applications as it imposes a limit on the duration of efficient biohydrogen production. One solution to overcoming this problem is the use of conventional photobioreactors to agitate and provide steady-state culture conditions. While these methods are useful they tend to require expensive equipment and are energy intensive (6).

A promising alternative to batch cultures is the use of non-growing immobilized bacteria adhered to a substrate in a matrix such as alginate, sol-gel, or latex (13, 13, 18, 23). Latex is a stable, nontoxic, nanoporous matrix useful for the immobilization of microbes and is preferable to alginate and sol-gel because it is adhesive, more economical, does not collapse upon drying, and can be used to immobilize a high concentration of cells (11). These solid supports are ideal for photosynthetic hydrogen production because they provide a high surface area to volume ratio for incident light, efficient rates of gas diffusion, and can increase cell longevity (11, 21). Thus, latex coatings have been successfully developed for hydrogen production using the purple non-sulfur phototroph *Rhodopseudomonas palustris* with stable, sustained rates of biohydrogen production (2.08 ± 0.01 mmoles H_2 m^{-2} day^{-1}) observed for 4000 hours (14, 15).

In addition to biohydrogen production, latex-based coatings have been used as mercury biosensors in *E. coli* (22), for the study of membrane-associated dehydrogenase reactions in *Gluconobacter oxydans* (10), and high temperature coatings have been developed for the study of amylase in *Thermotoga maritima* (21). While biocatalytic latex coatings have been utilized for a variety of applications, one of the challenges is that these coatings cannot be stored dry at ambient relative humidity and temperatures and maintain activity. Current methods for coating and storing *R. palustris* in latex at ambient temperatures require an environment with a 60% relative humidity in order to preserve the cells and maintain activity (14). While this strategy has proven successful for short-term storage, it is not practical for long-term portable storage for “off-the-shelf” hydrogen production applications.

The drying of cells imposes stress and results in metabolic and structural changes as they cope with the loss of water. As cells lose their water potential, membrane integrity is affected as the combination of osmotic stress and increases in the T_m of the membrane phase transition result in cell shrinkage and leaking of cytoplasmic components (5, 24). Furthermore, damage to proteins can be mediated by reactive oxygen species generated from lipid peroxidation and metal-catalyzed protein oxidations in iron-dependent Fenton reactions where ferryl ion combines with H_2O_2 to react with protein side chains

(12, 24). A reduced DNA water content also makes it more susceptible to alkylation, oxidation, crosslinking, depurination, and radiation (2, 17, 19, 24).

Despite the damaging effects of desiccation, some cells are able to minimize osmotic shock by accumulating disaccharides (5) as well as other mechanisms of desiccation tolerance that are often used concomitant with osmolyte regulation. Some of these strategies are to enhance the extracellular slime layer to minimize the rate of water loss (9), decrease metabolic activity to reduce the energy demand of the cell (7), maintain multiple copies of their genome (30), and produce histone-like DNA binding proteins to support nucleoid structure (25). Furthermore, some organisms minimize damage through the accumulation of water stress proteins (Wsp), as found in some cyanobacteria (28), while others minimize oxidative damage by incident UV through photoprotective pigments (4) and antioxidant enzymes (29).

In order to develop latex-embedded *R. palustris* cells as portable catalysts for biohydrogen production two of these strategies were utilized to improve desiccation tolerance; the addition of osmotic shock stabilizers and the minimizing of metabolic activity. Through the use of osmotic stabilizers such as glycerol, sucrose, trehalose, and sorbitol in the latex formulation, along with changes in temperature and humidity during the drying process we have identified conditions at which latex coatings of *R. palustris* can be preserved for long-term storage and maintain their ability to produce biohydrogen. Thus, we have developed a method to store biocatalytic latex coatings of *R. palustris* cells for use as "off-the-shelf" lightweight catalysts for generating biohydrogen.

Results

This study began by developing a technique to extensively screen nitrogen fixing bacteria isolated from unique environments suspected of H_2 production. In this work, a fairly new detection method was examined in which a H_2 color indicator was prepared. This color indicator consists of Wilkinson's catalyst and several redox sensitive dyes: methylene blue, crystal violet, neutral red, and methyl orange. Tris rhodium chloride, commonly known as Wilkinson's catalyst was sulfonated to make it water soluble and was subsequently added to vials containing growth media and one redox dye. H_2 (100%, 20%, 5%, 2%, 1%, and 0.5%) was added to each vial, while negative controls were flushed with argon, and incubated 18 h at room temperature. A change of color was observed in all test solutions containing 5-100% H_2 compared to the negative controls. Methyl orange appears to be most sensitive to hydrogen gas, since its visual change of color was detected even with 1% H_2. In addition, spectrophotometric scans (visible spectrum) of each dye/H_2 combination were conducted. We adapted the method as a high-throughput technique to screen bacterial H_2 production in 96-

well plates. The results were published in the Proceedings from the NHA annual Hydrogen conference (Appendex).

Bacterial isolates which tested positive for hydrogen production (five cyanobacteria and *Rhodopseudomonas palustris*, a genetically modified for increased hydrogen production obtained from U. Washington) were then absorbed onto latex coatings (developed by NC State U.) and the resulting mats were tested for hydrogen production for 18 days. All isolates produced hydrogen throughout the incubation period. Isolates obtained from the Cayman Islands and the Bahamas (isolates from hypersaline environments) were then tested using growth on N-free plates, colormetric assays for hydrogen production and positive isolates were embedded in latex mats for hydrogen assays. Although many isolates absorbed well the the mats, high salt content interfered with the latex stability.

A series of tests were then designed to test environmental conditions on the stability of inoculated latex mats. These experiments included the effects of humidity, added carbon source, pH, oxygen concentration, temperature and osmotic stabilizers on the effect of hydrogen production of stored inoculated latex mats. Anaerobic respiratory activity was measured for 84 days using confocal laser scanning microscopy of inoculated mats. These results were published in Microbial Biotechnology (Attachment)

Mixed cultures embedded in latex were also tested. In one experiment *Calothrix* was embedded with *Rhodopseudomonas*. The coatings exhibited a biphasic pattern, increasing after 8 days incubation. By combining these phototrophs that absorb different wavelengths of light, a greater fraction of natural light can be utilized to power hydrogen production.

The next approach in the development of a microbial fuel cell was to link hydrogen production with electricity generation by Bacteria embedded in latex mats. This concept incorporates H2 produced from photosynthesis by immobilized photosynthetic bacteria and electricity production by a second immobilized bacterial culture capable of linking H2 oxidation to electron transfer at an electrode. Results linked electricity production and photosynthetic H2 production. We found enhanced rates and increased quantities of electrons transferred from bacteria to solid phase terminal electron acceptors (including electrodes). We found that melanized bacterial mats generate higher currents than monmelanized mats. Hydrogen

production from photosynthetic bacteria was observed in conjunction with cell immobilization techniques using latex polymers. Latex immobilization was shown to increase bacterial longevity and activity, permit high cell density (and high reaction rates), and protect against mechanical degradation and contamination. Bacteria immobilized in latex remain viable for 3 months, possibly much longer and sustained bacterial H2 oxidation was also been demonstrated. Our concept for integrating these recent advancements will provide more information regarding enhanced modular hydrogen fueled microbial fuel cell linked to photosynthesis.

Summary

A technique to extensively screen nitrogen fixing bacterial isolates from unique environments suspected of H_2 production was developed. Wilkinson's catalyst and methyl orange was the most sensitive to hydrogen gas. Visual change of color was detected even with 1% H_2. A number of bacterial isolates were screened and found to be hydrogen producers, including from samples obtained from the Cayman Islands and the Bahamas. These isolates were absorbed onto latex mats and then mats tested for hydrogen production. The most productive hydrogen producers embedded in latex were cyanobacteria and nonsulfur phototrophs.

The ability to store these coatings under modified drying conditions was investigated in this study. Initial experiments reinforce the use of osmoprotectants, such as glycerol, sucrose, sorbitol, and trehalose, for cell viability during the drying process. While there was no major difference between the ability of the various stabilizers to protect the cells, sorbitol was found to be the least protective over long-term storage. Furthermore, storage of coatings at 4 ºC versus 22 ºC did not improve the recovery of biohydrogen production over time regardless of the stabilizer. There were, however, significant differences in the hydrogen production ability of coatings stored at different humidities. Over long-term storage for up to 8 weeks, coatings stored at a low moisture content (< 5% humidity) were able to recover and produce biohydrogen upon hydration while those stored at 60% humidity were not able to recover, even after only 4 weeks of room temperature storage. The storage of coatings with osmotic stabilizers and at low humidity improves the practical value of this technology as an "off-the-

shelf" biocatalyst for biohydrogen production and extends the use of these coatings for other organisms and applications. Mixed phototrophic cultures embedded in latex were found to be superior for hydrogen production, and when mixed with melanized cultures resulted in increased quantities of electrons transferred from bacteria to solid phase terminal electron acceptors, thus producing electricity in the fuel cells.

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