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Hydrogen Production by Photoreactive Nanoporous Latex Coatings of Nongrowing *Rhodopseudomonas palustris* CGA009

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Nonuniform light distribution is a fundamental limitation to biological hydrogen production by phototrophic bacteria. Numerous light distribution designs and culture conditions have been developed to reduce self-shading and nonuniform reactivity within bioreactors. In this study, highly concentrated (2.0×10^8 CFU/μL formulation) nongrowing *Rhodopseudomonas palustris* CGA009 were immobilized in thin, nanoporous, latex coatings. The coatings were used to study hydrogen production in an argon atmosphere as a function of coating composition, thickness, and light intensity. These coatings can be generated aerobically or anaerobically and are more reactive than an equivalent number of suspended or settled cells. *Rhodopseudomonas palustris* latex coatings remained active after hydrated storage for greater than 3 months in the dark and over 1 year when stored at −80 °C. The initial hydrogen production rate of the microphotobioreactors containing 6.25 cm^2, 58.4 μm thick *Rps. palustris* latex coatings illuminated by 34.1 PAR μmol photons m^{-2} s^{-1} was 6.3 mmol H_2 m^{-2} h^{-1} and had a final yield of 0.55 mol H_2 m^{-2} in 120 h. A dispersible latex blend has been developed for direct comparison of the specific activity of settled, suspended, and immobilized *Rps. palustris*.

1. Introduction

It has been estimated that approximately 80% of the world's energy demand is met through the use of fossil fuels (*1*). Alternative energy sources are needed because global energy demands are exponentially increasing and fossil fuel availability will become increasingly limited. Direct conversion of solar energy to hydrogen by microbes is an attractive alternative energy source for fuel cells. The first report of biological hydrogen production was made over 110 years ago (*2*, reviewed in *3*). Various applications of photosynthetic organisms (bacteria, cyanobacteria, and algae) for biohydrogen production have been studied for the past 60 years; however, a large-scale industrial process has not been developed. Biohydrogen as an alternative energy resource has been extensively reviewed in the literature (*1*, *3*−*6*).

Nitrogenase-mediated hydrogen production in phototrophic purple bacteria depends on the transfer of electrons from organic electron donors to reduced electron carriers and the generation of ATP from photosynthesis. The reduced electron carriers are used in the conversion of dinitrogen gas to ammonia catalyzed by the nitrogenase complex with ATP. Establishing a nongrowth system by nitrogen starvation allows the nitrogenase enzymes to forego their primary reaction mechanism of dinitrogen reduction and solely reduce protons to hydrogen. Even though the nitrogenase enzymes require more energy and have a lower rate of hydrogen production than hydrogenases, the advantages of using *Rhodopseudomonas palustris*, a purple non-sulfur bacterium, may outweigh the disadvantages. These organisms have recently been proposed as part of an integrated hydrogen production approach with green algae for efficient light absorption (*7*). Photoheterotrophic organisms such as *Rps. palustris* are capable of utilizing a wide range of light wavelengths as well as electron donors. This translates into a higher number of potential genetic targets for optimization and numerous electron donor sources for hydrogen production (*8*, *9*).

Most photobioreactors are unable to uniformly illuminate microbes without elaborate light distribution systems and are inefficient when scaled up. One major limiting factor to scaling up is the rapid reduction in the ratio of illuminated surface area to internal shaded volume in large suspension or tubular photobioreactors, compared to bench-scale photobioreactors of similar geometry (*10*). Hydrogen production using multilayered plate reactors or thin photoreactive coatings have the potential to dramatically increase volumetric reactivity, stabilize the embedded microbes, and overcome the difficulty of uniform illumination of photoreactive microbes at high cell densities (*11*).

Many immobilization methods have been applied to photosynthetic organisms to stabilize and increase the rate of microbial biohydrogen production (*12*−*14*). Although previous immobilization methods were successful to varying degrees, they did not demonstrate one or more of the following: a coating that is adhesive, immobilization of highly concentrated microbes (100- to 1000-fold concentration), use of an inexpensive immobilization matrix, preservation of the viability of the immobilized microbes after being dried at ambient temperature or activity after frozen storage. In addition to satisfying the

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above conditions this work is different from all of the previously published immobilized photosynthetic microbial literature in that we have demonstrated the utility of an adhesive nonhydrogel matrix (nanoporous latex coating) which can be formulated to contain *Rps. palustris* at a biomass load of at least 50% of the wet volume.

Biocatalytic latex coatings are adhesive, mechanically stable, nanoporous matrices useful for the immobilization of microbes (*15*−*17*). Immobilization in thin latex coatings stabilizes microbial activity, concentrates the biocatalyst to increase volumetric reactivity (bioprocess intensity) and can protect the microorganisms from mechanical degradation and deactivation (*17*). An advantage to using the thin (<100 μm) latex coatings for photosynthetic microorganisms is the reduction of self-shading and minimization of diffusion resistance. The coatings will also facilitate the study of the rate-limiting biochemical reactions of light utilization and electron transport necessary for optimizing biohydrogen production. Martens and Hall were the first to report the immobilization of *Synechococcus* sp. in a latex coating; however, the coatings were not optimized beyond the selection of the methylmethacrylate and *n*-butyl acrylate copolymer latexes (*14*).

The aim of this study is to develop a microphotobioreactor containing *Rps. palustris* in a latex coating that could be used to address two of the major hindrances to efficient biohydrogen production from purple bacteria. The first is inefficiency of light distribution (uniform illumination), and the second is low light utilization efficiencies at high light intensity (photosaturation). A simple, inexpensive model microphotobioreactor (MPB) was developed containing a single translucent adhesive layer of nanoporous latex containing concentrated nongrowing *Rps. palustris* CGA009 for uniform light distribution. *Rhodopseudomonas palustris* CGA009 contains three functional nitrogenase isozymes and does not express an uptake hydrogenase due to a frameshift in the *hupV* regulatory gene (*18*, *19*). The MPBs were used in this study to analyze the initial rate of hydrogen production as a function of coating formulation, thickness, and light intensity per square meter. We demonstrate that these adhesive coatings facilitate uniform illumination, are highly nanoporous, can retain 90% viability following drying, and remain active after hydrated storage for over 3 months in the dark at 30 °C or greater than 1 year frozen at −80 °C.

2. Materials and Methods

2.1. Bacterial Strain, Growth Conditions, Media, and Latex. *Rhodopseudomonas palustris* wild type CGA009 was grown anaerobically in 160 mL glass serum bottles (Wheaton, Millville, NJ) containing 100 mL of nitrogen fixing photosynthetic medium [PM(NF)] (*20*) with an initial headspace pressure of 1 atm N_2. (All gases used in this study were from Minneapolis Oxygen, Minneapolis, MN.) Cultures were incubated at 31 ± 2 °C, in an incubator (Hotpack Corp., Philadelphia, PA) under 34.1 ± 2.0 PAR μmol photons m^{-2} s^{-1} (LI-COR, LI-190SA Quantum Sensor, Lincoln, NE , $n = 22$ over 6 months, 26.7 cm below three 60 W clear incandescent lights) without shaking. PM(NF) was composed of (per liter) 25 mL 0.5 M Na_2HPO_4, 25 mL 0.5 M KH_2PO_4, 1 mL 0.1 M sodium thiosulfate, 1 mL 2 mg/mL *para*-aminobenzoic acid, 20 mL 1 M sodium acetate, 1 mL concentrated base solution. Concentrated base solution was composed of (per liter) 20 g nitrilotriacetic acid, 28.9 g $MgSO_4$, 6.67 g $CaCl_2 \cdot H_2O$, 18.5 mg $(NH_4)_6Mo_7O_{24} \cdot 7H_2O$, 198 mg $FeSO_4 \cdot 7H_2O$, 100 mL metal 44 solution. Metal 44 solution was composed of (per liter) 2.5 g EDTA (free acid), 10.95 g $ZnSO_4 \cdot 7H_2O$, 5.0 g $FeSO_4 \cdot 7H_2O$, 1.54 g $MnSO_4 \cdot H_2O$, 392 mg $CuSO_4 \cdot 5H_2O$, 250 mg $Co(NO_3)_2 \cdot 6H_2O$, 177 mg $Na_2B_4O_7 \cdot 10H_2O$, and a few drops of concentrated H_2SO_4 to prevent precipitation.

All latexes were obtained from Rohm and Haas Co. (Philadelphia, PA). SF091 prepared without biocides is a commercially available poly(vinyl acetate-*co*-acrylate) *co*-polymer latex (VAA) with hydroxyethylcellulose grafted onto the polymer particle surface, an average particle size of 280 nm, and T_g ~10 °C; the pH was adjusted to 7.0 prior to use. JP922 is a biocide-free poly(butyl acetate-*co*-vinyl acrylate) *co*-polymer latex (BAVA) with hydroxyethylcellulose grafted onto the polymer particle surface, an average particle size of 292 nm, and T_g ~7 °C; the pH was adjusted to 7.0. JP917 is a biocide-free poly(butyl acetate-*co*-vinyl acrylate) *co*-polymer latex (BAVA) without hydroxyethylcellulose, an average particle size of 324 nm, and T_g ~7 °C; the pH was adjusted to 7.0.

2.2. Headspace Analysis by Gas Chromatography and Initial Rate Calculations. Headspace analysis was accomplished using a Hewlett-Packard 5890 Series II gas chromatograph containing a 7 ft × 1/8 in. i.d. Haysep DB 100/120 porous mesh polymer packed stainless steel column and a thermal conductivity detector. Argon was used as the carrier at a flow rate of 6 cm^3/min. Injector/oven/detector temperature settings of 65/65−110−65 (10 °C/min ramp-cycle)/150 °C resulted in detection and resolution of hydrogen and carbon dioxide with a 15 min sample runtime.

The moles of hydrogen (X_{H_2}) present in the argon (X_{Ar}) headspace of the MPB were calculated using the equation $X_{H_2} = (X_{Ar}(C_{H_2}))/(1 - C_{H_2} - C_{CO_2})$. The concentration of hydrogen (C_{H_2}) and carbon dioxide (C_{CO_2}) in the sample were expressed as mole fractions calculated using standard curves generated from triplicate injections of each gas. Corrections were made for the number of moles of each gas removed at sampling for cumulative hydrogen production curves. The headspace over the 10 mL of media in the Balch tubes was 16.5 cm^3 and the initial pressure was 1 atm of argon at 30 °C. Because of the low solubility of hydrogen in water under these conditions, a small amount of hydrogen (~1% of the moles of H_2 in the headspace) is dissolved in the 10 mL of media (*27*). The dissolved hydrogen was not taken into account in our hydrogen production calculations.

Experiments were performed in triplicate using 6.25 cm^2 latex coatings (unless otherwise noted) removed from the same template. Initial rates were determined using a linear fit over the first 70−80 h (4 pts) of each experiment. The 2680 h storage experiment was fit for the 2850−3500 h points representing the linear rate of hydrogen production attained by those MPBs excluding the lag period.

2.3. Preparation of *Rps. palustris* Latex Coating Microphotobioreactors. *2.3.1. Latex Coating Template.* The following method was used to generate 6.25 cm^2 latex coating MPBs (Figure 1A). A 20 cm × 20 cm glass plate was covered by 19 mm wide nonoverlapping double stick tape (3M, St. Paul, MN). A 125 μm thick polyester sheet (DuPont Melinex 454, Tekra Corp, NJ) precut (20 cm × 20 cm) with parallel 14 cm lines separated by 1 cm was overlaid onto the glass plate perpendicular to the tape. After the polyester was adhered to the glass support, the premarked cross lines (parallel lines [Blue] 13.3 cm apart, perpendicular to and centered on the 14 cm lines, [RED]) were cut with a razor blade allowing for removal of the strips after coating. Finally a 20 cm × 20 cm adhesive vinyl mask (84 ± 4 μm thick Con-Tact, Stamford, CT) was cut to have a 12.5 × 11 cm square opening. This polyester mask was overlaid such that each strip (13.3 × 1 cm) was centered on it

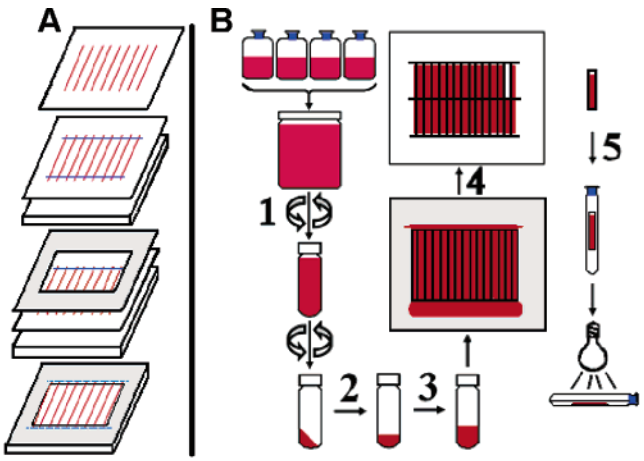

Figure 1. Template design and coating method for MPBs. (A) Parallel cuts (red lines) 14 cm long and 1 cm apart in polyester substrate. After polyester was adhered to double stick tape on the glass support, perpendicular cuts (blue lines) were made 13.3 cm apart. Finally a precut Con-Tact mask (grey) with the inner dimensions of 12.5 cm by the number of strips + 1 cm was overlaid onto the polyester. Fully assembled template on glass plate. (B) 1. Centrifugation of photosynthetic, diazotrophically grown *Rps. palustris* CGA009. 2. Addition of sucrose and glycerol to wet cell pellet with mixing. 3. Addition of latex to cell formulation with mixing. 4. Mayer rod draw down and drying of *Rps. palustris* CGA009 photoreactive coatings. 5. Removal of mask and individual 6.25 cm^2 coatings, insertion of coating into Balch tubes, argon flush, and illumination of assembled MPB.

to generate an effective coating area (area exposed through mask) of 12.5 cm^2. This method yields a template for 20 identical 6.25 cm^2 latex coatings for MPBs.

2.3.2. Formulation. *Rhodopseudomonas palustris* was grown to an approximate OD_{660} of 0.8 corresponding to early stationary phase. The methods described below were performed anaerobically including the drying of the coating in an anaerobic hood with temperature and humidity control (described below). Culture broth was centrifuged (Beckman JA-10 rotor) in widemouth sealing bottles (Fisher Scientific, Fairlawn, NJ) at 4,424 × *g* and 4 °C for 15 min. Cell pellets were resuspended in <40 mL PM(NF) without acetate and transferred to sealing Oakridge centrifuge tubes (Fisher Scientific, Fairlawn, NJ). Cell suspensions were centrifuged in a bench top centrifuge (Centra-4 centrifuge, International Equipment Company, Needleham Heights, MA) at 5,000 × *g* and room temperature for 15 min (Figure 1B). The latex emulsion was prepared as previously described (*15*, *17*) using the formulation ratio 1.2 g wet cell weight (WCW), 350 μL 0.58 g/mL sucrose, 150 μL 100% glycerol, and 1 mL SF091 (*15*). The viscosity of the above *Rps. palustris* formulation varied from 0.90 to 0.02 Pa·s as the shear rate increased from 0.75 to 5,100 s^{-1} (Ares-LS, TA Instruments, New Castle, DE).

2.3.3. Anaerobic Coating Method. A Coy anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI) retrofitted with a dehumidifier and humidifier, which controlled relative humidity (RH) (monitored by a calibrated digital temperature and RH sensor model TM125, Dickson, Addison, IL), was used for all culture transfers and coating steps unless otherwise noted. The chamber contained a maximum of 5% hydrogen with the balance argon.

The *Rps. palustris* coating formulation was transferred using a pipet onto the mask of the assembled template and drawn down by hand (Figure 1, B4) with a 26 wire wound Mayer rod (Paul N. Gardner Co., Pompano Beach, FL). The coating was dried for 1 h at a relative humidity of 62 ± 2% at 32 ± 2 °C (Dickson TM125 calibrated digital temperature/humidity sensory, Addison, IL). After drying, the mask was removed, and the 12.5 cm coatings were cut in half to generate the final 6.25 cm^2 coatings. The individual coatings were placed in 18 mm × 150 mm Balch tubes (Bellco Biotechnology Inc., Vineland, NJ) containing 10 mL of PM(NF) medium with 20 mM acetate unless otherwise indicated to create MPBs. The MPBs were sealed with butyl rubber septum stoppers and aluminum crimp caps (Bellco Biotechnology Inc., Vineland, NJ) and then removed from the hood and flushed with pure argon for 30 min. The flushed tubes were allowed to pressurize with approximately 30 psi of argon so they could be uniformly vented using a water trap to equilibrate to 1 atm. The latex coating thickness and permeability were determined as previously described (*15*, *17*, *21*). Strips with varying total surface area were prepared from the 1 cm × 12.5 cm coatings by cutting with a razor.

2.3.4. Aerobic Coating Method. The *Rps. palustris* coating formulation was pipetted onto the assembled template and drawn down with a 26 wire wound Mayer rod using a constant pressure drawdown coating apparatus (Paul N. Gardner Co., Pompano Beach, FL). The RH and temperature during drying was >46% and 22 ± 1 °C, respectively. Aerobically prepared MPBs were assembled as described for the anaerobic coatings.

Settled cell controls were made by mixing the emulsion as described above with 1 mL of PM(NF) media (without a carbon source) in place of the 1 mL of latex. A 52.3 μL aliquot of the modified emulsion was transferred to a Balch tube containing 10 mL of PM(NF) medium with 20 mM acetate.

2.4. Cell Formulation and Coating Viability. The cell formulation viability was determined by triplicate CFU plate counts of serial dilution assays from coatings made with either JP917 or the 40% SF091−60% JP922 blend formulation. The wet emulsion volume in a single 12.5 cm^2 coating was determined from replicate aerobic coatings generated as described above with the following exceptions. The template consisted of a preweighed 3 cm × 15 cm polyester sheet with a Con-Tact mask made for one 1 cm × 12.5 cm coating. Using a 26 wire wound Mayer rod and 175 μL of emulsion, 13 replicate coatings were made. Immediately the mask was removed, and the polyester with the wet coatings was weighed. The volume of emulsion was determined from a standard curve generated by weighing tared 200 μL pipet tips (Fisherbrand Redi-Tip, Fisher Scientific, Fairlawn, NJ) containing 75, 100, 125, or 175 μL of emulsion. Weight was determined with four or more replicates except for 175 μL, which had two. Each pipet tip was wiped free of any emulsion left on the outside of the tip prior to being weighed. The number of viable cells necessary for the settled cell controls was calculated using the cell formulation viability and the wet coating volume.

2.5. Coating Composition. MPBs were made in triplicate from coatings generated with formulations containing both sucrose and glycerol, each individually, or without pore-forming additives. Hydrogen production was monitored (data not shown) for each coating formulation incubated in the MPBs without 20 mM acetate in the PM(NF) medium. For a qualitative measure of coating porosity hydrogen production in the absence of 20 mM acetate was subtracted from the MPBs incubated with 20 mM acetate. In addition to the coatings, a settled cell MPB (containing sucrose and glycerol) was incubated with and without 20 mM acetate.

2.6. Disruption of Dispersible Coatings, Coating Thickness, and Light Intensity. Triplicate coatings were disrupted for each measurement. The entire MPB tube was sonicated for 5 min in a Branson 1200 bath sonicator and then vortexed for 1 min. This was repeated three times for complete dispersion.

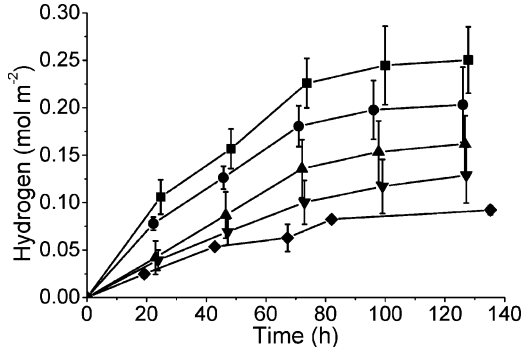

Figure 2. Hydrogen production from 20 mM acetate by *Rps. palustris* latex coatings generated using alternate formulations. Each condition has been normalized to the respective triplicate MPB that did not contain 20 mM acetate: glycerol + sucrose (■); sucrose only (●); glycerol only (▲); no pore forming additives (▼); settled cells (◆). Error bars ± 1 SD, $n = 3$.

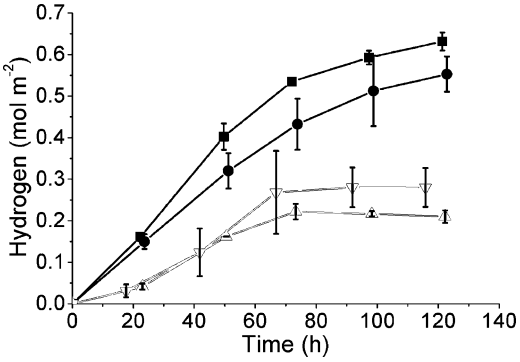

Figure 3. Hydrogen production initial rate and final yield of aerobically and anaerobically generated coatings incubated either aerobically or anaerobically. The coating method does not affect hydrogen production when hydrogen production is anaerobic; however, hydrogen production is inhibited under aerobic conditions (△,▽). Aerobic coating, anaerobic incubation (■); anaerobic coating, anaerobic incubation (●); anaerobic coating, aerobic incubation (▽); aerobic coating, aerobic incubation (△). Error bars ± 1 SD, $n = 3$.

The coatings of different thicknesses were generated aerobically on four separate templates. Two assembled coating templates were used without a vinyl mask and drawn down using either a 0 (bare rod) or 26 wire wound Mayer rod. The third coating was drawn with a normal template (see section 2.3.1 above) using a 26 wire wound Mayer rod. The last template had four layers of vinyl mask and was drawn down using a 78 wire wound Mayer rod. High light intensity MPBs were incubated at 31 ± 2 °C under six 200 W incandescent lights at 355.5 ± 14.5 PAR μmol photons m^{-2} s^{-1}.

3.0 Results and Discussion

3.1. Viability of *Rps. palustris* Latex Formulation. SF091 latex prepared without biocide was selected for use in this study because of its previous use in biocatalytic coatings of Gram-negative microorganisms (*15*, *17*, *21*). The viability of the cell formulation using SF091 latex prior to drying was $2.03 \pm 0.43 \times 10^8$ CFU/μL, which is approximately 100-fold more concentrated than the growth culture ($1.20 \pm 0.06 \times 10^6$ CFU/μL).

3.2. *Rps. palustris* Latex Coating Formulation. Sucrose and glycerol are known to increase nanoporosity and viability, respectively, when added to biocatalytic coatings containing living microorganisms (*15*, *17*). Formulations with different combinations of the pore-forming additives were made to determine the effects of sucrose and glycerol on hydrogen production. Sucrose and glycerol are not hydrogen production substrates under nongrowing conditions (data not shown). Hydrogen production however was observed to be a function of coating composition (Figure 2). The coatings that contained both sucrose and glycerol had the highest hydrogen evolution rates followed by the sucrose-only coatings. The glycerol-only coatings and the coatings containing no pore-forming additives had an even lower initial rate and final yield. The settled *Rps. palustris* cells had the lowest initial rate and final yield. This may be explained by the loss in reactivity due to nonuniform illumination of the settled cells. Immobilization in the coating facilitates uniform light distribution, resulting in an increase in hydrogen production. The increasing rates and yields of hydrogen production with the addition of sucrose and glycerol are likely due to an increase in porosity of the coating and the viability of immobilized *Rps. palustris*. The focus of our ongoing research is to determine the degree to which these two factors impact hydrogen production so that coatings can be engineered with optimal porosity, viability, and optical properties.

3.3. Aerobic Coating Method. In addition to the anaerobic coating method, an aerobic coating method would be useful for rapid evaluation of hydrogen production kinetics and to determine the in vivo stability of nitrogenase mutants. However, nitrogenases are rapidly inactivated by oxygen, resulting in inhibition of hydrogen production (*3*, *22*). Figure 3 is a comparison of hydrogen production from coatings made using aerobic and anaerobic coating methods. Coatings measuring 6.25 cm^2 generated and dried aerobically (air) or anaerobically (5% hydrogen in argon) with humidity control were incubated in either aerobic (air) or anaerobic (argon) environments. As anticipated, hydrogen production was inhibited when an aerobic headspace was present. However, no loss of activity was observed between the two coating methods when an argon atmosphere was maintained during hydrogen production. The ability to coat *Rps. palustris* aerobically greatly increases the versatility of this coating method because it eliminates the restriction of making coatings in an anaerobic chamber.

3.4. Frozen Storage. Frozen storage of *Rps. palustris* latex coatings would be useful for transporting coatings in a stable inactive state. This was tested by illuminating *Rps. palustris* latex coatings after increasing durations of storage at −80 °C frozen in 25% glycerol PM(NF) medium without acetate (Figure 4). Even after frozen storage for over 1 year, the photoreactive coatings were found to retain ∼75% of the hydrogen production initial rate compared to fresh coatings.

3.5. Hydrogen Production as a Function of Light Intensity and After Storage at 30 °C. In order to determine whether hydrogen production is light-dependent for nongrowing *Rps. palustris*, MPBs were stored in the dark at 30 °C (foil-covered tubes) and illuminated (removal of foil covering) after dark storage (Figure 5). Hydrogen accumulation does not occur in the absence of light. The initial rate of hydrogen production decreased after 138 h of hydrated dark storage at 30 °C; however, further storage to 349 h in the dark does not result in a further decrease in the initial rate. After 2680 h there is a significant lag before hydrogen production (1.4 mmol m^{-2} h^{-1} rate) was observed, which was >20% of the initial observed when fresh coatings were illuminated (6.3 mmol m^{-2} h^{-1}).

The effect of higher light intensities on hydrogen production initial rate was investigated. Increasing the light intensity from 34.1 ± 2.0 PAR μmol photons m^{-2} s^{-1} to 355.5 ± 14.5 PAR μmol photons m^{-2} s^{-1} increased the hydrogen production initial rate by 15% (Figure 6) indicating *Rps. palustris* is photosaturated under the high light intensity condition.

3.6. Hydrogen Production as a Function of Coating Thickness. Hydrogen production was investigated for aerobi-

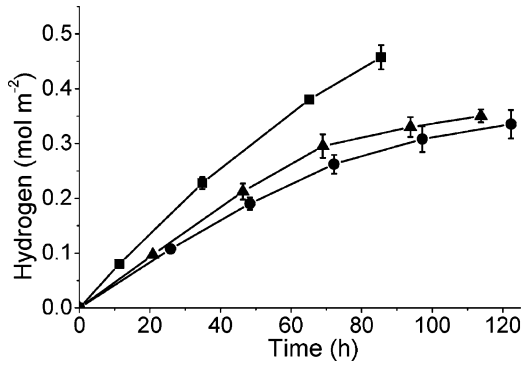

Figure 4. *Rhodopseudomonas palustris* latex coatings illuminated after frozen storage in the dark at −80 °C. After 330 days (●) and 384 days (▲) ∼75% of hydrogen production initial rate is retained compared to fresh coatings (■). Error bars ± 1 SD, $n = 3$.

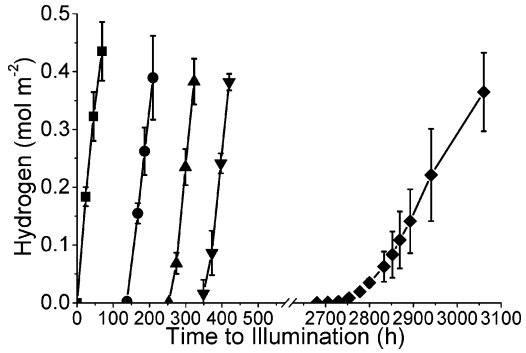

Figure 5. *Rhodopseudomonas palustris* latex coatings illuminated after hydrated storage in the dark (foil-covered MPBs) at 30 °C. Following 2680 h of dark storage at 30 °C the MPBs retain >20% of the fresh coating (no storage) initial rate. The time to illumination (h) and initial rate of hydrogen production (mmol H_2 m^{-2} h^{-1}): (■) 0, 6.3; (●) 138, 5.3; (▲) 253, 5.6; (▼) 349, 5.3; (◆) 2680, 1.4. Error bars ± 1 SD, $n = 3$.

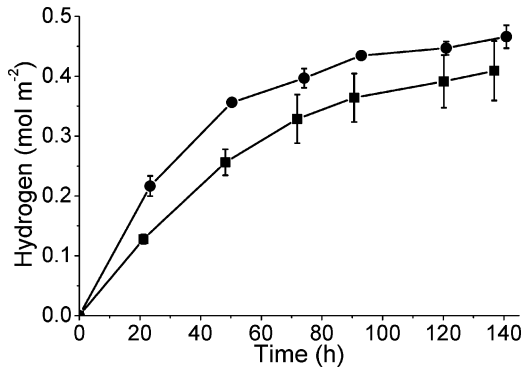

Figure 6. Hydrogen production by *Rps. palustris* latex coatings incubated under low and high-intensity light. Hydrogen production initial rate increases when *Rps. palustris* latex coating MPBs are incubated under 355.5 ± 14.5 μmol photons m^{-2} s^{-1} (●) compared to 34.1 ± 2.0 μmol photons m^{-2} s^{-1} (■). Error bars ± 1 SD, $n = 3$.

cally generated coatings with a range of thickness from 10 to 250 μm to estimate coating specific activity under constant illumination (34.1 ± 2.0 PAR μmol photons m^{-2} s^{-1}). As expected, hydrogen production initial rate and final yield increased with thickness, although coating specific activity (mmol H_2 m^{-2} h^{-1} μm^{-1}) did not (Figure 7). The highest coating specific activity was 0.074 mmol H_2 m^{-2} h^{-1} μm^{-1} for the 40.9 ± 1.9 μm coating. Thinner coatings (10.5 ± 3.6 μm, 0.038 mmol H_2 m^{-2} h^{-1} μm^{-1}) were limited by catalyst concentration (*Rps. palustris*), and the thicker coatings (84.2 ± 6.1 μm, 0.054 mmol H_2 m^{-2} h^{-1} μm^{-1} and 231.2 ± 41.5 μm, 0.032 mmol H_2 m^{-2} h^{-1} μm^{-1}) were likely limited by light scattering that restricted light penetration to the bottom of the coating. Comparing the coating thickness as a function of coating specific activity indicates under these conditions that an optimal coating thickness for a single layer of *Rps. palustris* CGA009 is ∼50 μm.

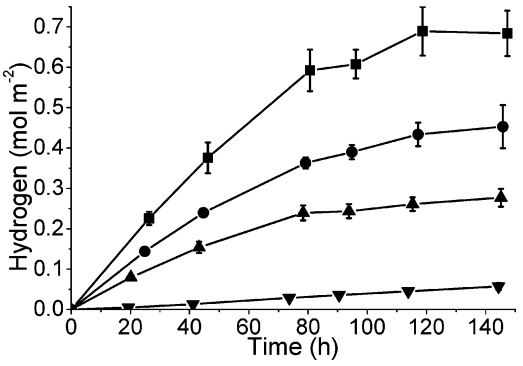

Figure 7. Hydrogen production by *Rps. palustris* latex coatings of increasing thicknesses. Hydrogen production increased with coating thickness. Coating specific activity 0.074 mmol H_2 m^{-2} h^{-1} μm^{-1} was highest for the 40.9 μm thick coating (▲). Coating thickness (μm), initial rate (mmol H_2 m^{-2} h^{-1}): (■) 231.2 ± 41.5, 7.3; (●) 84.2 ± 6.1, 4.5; (▲) 40.9 ± 1.9, 3.0; (▼) 10.5 ± 3.6, 0.4. Error bars ± 1 SD, n = 3.

3.7. Hydrogen Production Specific Activity in Dispersible Coatings. Although the viability of *Rps. palustris* in SF091 (VAA) formulations can be determined prior to drying, the coatings were too stable (coalesced) after drying to be completely disrupted to release single cells by mild sonication and vortexing (*15*, *17*). A cell formulation using JP917 (BAVA) latex was used instead to generate coatings that dispersed upon rehydration. The JP917 *Rps. palustris* coatings were made as described for SF091 aerobic coatings. Viability was determined from triplicate coatings after 15, 20, 30, 45, and 60 min of coat drying during which time the viability was constant (data not shown). The viability for the entire drying time ($n = 15$) was 1.8 ± 0.16 × 10^8 CFU/μL latex emulsion, which is ∼90% retention of the viability of the uncoated cell formulation. These dispersible (BAVA) coatings allowed for more accurate and reproducible determination of viability during the drying process but were not stable following rehydration and therefore could not be used for long-term hydrogen production initial rate studies.

What was needed was a coating that was stable when rehydrated for hydrogen production that could, however, be dispersed by mild sonication for determining cell viability. JP922 (BAVA) was used to make a dispersible polymer blend (40% JP922 and 60% SF091 v/v) with SF091 (VAA) that remained adhesive and intact for >1,000 h (data not shown) when rehydrated. These stable polymer blend (BAVA/VAA) coatings were dispersed by mild sonication and vortexing to determine the specific activity of latex immobilized *Rps. palustris* compared to suspended and settled cells. These dispersible BAVA/VAA coatings allowed more accurate and reproducible determination of viability after hydrogen production initial rate studies. Figure 8 shows the results of hydrogen production from BAVA/VAA immobilized, suspended, and settled *Rps. palustris*. The number of viable cells (CFU × 10^8/μL) present in each of the MPBs ($n = 3$) were 1.8 ± 0.2 immobilized, 1.5 ± 0.1 suspended and 1.2 ± 0.2 settled. The specific activities (mmol H_2 m^{-2} 10^{13} CFU^{-1} h^{-1}) were 6.4 for the immobilized cells and 5.4 for both the suspended and settled cells.

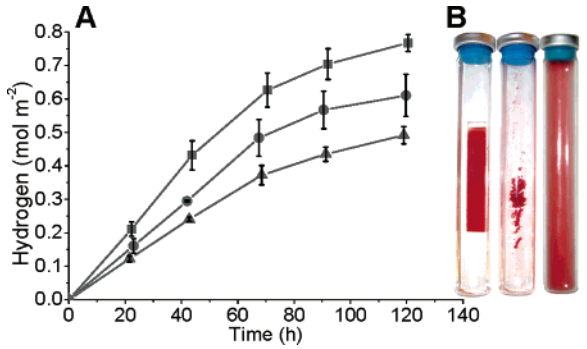

Figure 8. Hydrogen production by dispersible *Rps. palustris* BAVA/VAA latex blend coatings compared to suspended and settled cells. (A) Dispersible BAVA/VAA latex blend coated *Rps. palustris* (■) hydrogen production initial rate 9.0 mmol H_2 m^{-2} h^{-1}, yield 0.78 mol m^{-2}; suspended cell (●) hydrogen production initial rate 7.2 mmol H_2 m^{-2} h^{-1}, yield 0.61 mol m^{-2}; settled cell (▲) hydrogen production initial rate 5.4 mmol H_2 m^{-2} h^{-1}, yield 0.49 mol m^{-2}; Error bars ± 1 SD, n = 3, initial rate calculated from 0 to 70 h. (B) Left to right: immobilized *Rps. palustris* in dispersible BAVA/VAA latex blend coating; settled; suspended *Rps. palustris*.

4. Conclusions

Immobilization of photosynthetic bacteria has been reported to increase hydrogen production when compared to similar nonimmobilized conditions (*12*, *23*). This is the first report of an immobilized photoreactive bacterial system that not only concentrates *Rps. palustris* 100-fold in an adhesive latex coating but also retains activity after being dried at ambient temperatures, hydrated at 30 °C in the dark for >3 months, or stored frozen in 20% glycerol for greater than 1 year at −80 °C.

Immobilization of viable nongrowing *Rps. palustris* in 58.4 ± 6.8 μm thick, nanoporous latex coatings results in an increase in hydrogen production initial rate and final yield compared to that of suspended and settled *Rps. palustris*. Hydrogen production is light-dependent, and MPBs containing latex coatings produce hydrogen after 2680 h of hydrated dark storage at 30 °C. Despite the sensitivity of nitrogenase to oxygen, aerobically generated coatings retain nitrogenase activity and simplify preparation of multiple uniform coatings by eliminating the restriction of generating coatings in an anaerobic environment. This method is a useful technique for the rapid evaluation of mutants because the coatings can be made aerobically and stored hydrated.

The recent development of a dispersible BAVA/VAA latex polymer blend is a significant advancement in immobilization technology for adhesive nanoporous latex coatings for preserving the viability of cells at ambient temperature. The latex blend allows for both hydrogen production from a stable coating and dispersion of the same coating for further analysis of the latex-entrapped cells. Previous to this blend method, moderate disruption was used; however, this generated large clumps of latex containing many cells that readily settled (*17*). The latex blend method results in more reproducible recovery of most of the individual cells free of adherent adhesive latex particles for more accurate determination of viability by plate counting (CFU). Microscopy studies are now under way to further investigate the dispersion of this latex blend as well as other formulations. Indirect methods such as vital stains and ATP measurement to estimate the number of viable cells have been shown to be not sufficiently accurate for determination of coating specific activity (*17*). With this dispersible adhesive coating method, the activity of immobilized *Rps. palustris* can be directly compared to suspended or settled cells.

We have shown that a simple method to immobilize and store *Rps. palustris* in thin adhesive coatings results in an increase in the rate of hydrogen production as well as the final yield compared directly to the same number of settled or suspended cells. An in-depth comparison of this system to larger photobioreactors has not been made because of the small scale of these coatings. Numerous authors have previously compared hydrogen production rates among different organisms and reactor designs (*6*, *8*, *24*). The hydrogen production rate from *Rps. palustris* SF091 latex coatings is lower than that of other immobilized purple bacteria; however, the volumetric reactivity (1,840 mL H_2 L^{-1} h^{-1}) is comparable (*24*). The volumetric reactivity is a direct reflection of both the high cell density and the thinness of the coating. The lower rate of hydrogen production would be expected considering that in vivo nitrogenase activity of *Rps. palustris* is relatively low compared to other members of the *Rhodospirillaceae* family (*25*).

The advantages of thin adhesive nanoporous latex coatings (nontoxic adhesive matrix, dry/frozen viability, and long-term hydrated dark storage) could be applied in a variety of multilayer coating configurations. An example would be a stable integrated, thin, biological photoabsorber containing co-immobilized layers of different photosynthetic microbes each capable of capturing a different portion of the sunlight spectrum (*7*). Using this type of configuration the affects of photosaturation under high light intensities may be reduced by capturing and utilizing more of the available photons per square meter.

Studies are now under way to address low light utilization at high light intensities by screening carotenoid and photopigment mutants of *Rps. palustris* immobilized in latex coatings. These mutants will be used to increase the specific activity of multilayer photoreactive coatings (*26*). Multilayer coatings may be useful to increase the efficiency of total light utilization per square meter of absorbed sunlight by spatially immobilizing *Rps. palustris* pigment mutants in individual nanoporous polymer layers (*11*, *26*). The advantages of uniform light distribution and the multilayer immobilization of mutants engineered for absorption are expected to be synergistically beneficial in reducing photosaturation per square meter and, as a result, increasing the rate and final yield of hydrogen production.

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