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Preservation of H₂ production activity in nanoporous latex coatings of *Rhodopseudomonas palustris* CGA009 during dry storage at ambient temperatures

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Summary

To assess the applicability of latex cell coatings as an “off-the-shelf” biocatalyst, the effect of osmoprotectants, temperature, humidity and O₂ on preservation of H₂ production in *Rhodopseudomonas palustris* coatings was evaluated. Immediately following latex coating coalescence (24 h) and for up to 2 weeks of dry storage, rehydrated coatings containing different osmoprotectants displayed similar rates of H₂ production. Beyond 2 weeks of storage, sorbitol-treated coatings lost all H₂ production activity, whereas considerable H₂ production was still detected in sucrose- and trehalose-stabilized coatings. The relative humidity level at which the coatings were stored had a significant impact on the recovery and subsequent rates of H₂ production. After 4 weeks storage under air at 60% humidity, coatings produced only trace amounts of H₂ (0-0.1% headspace accumulation), whereas those stored at <5% humidity retained 27-53% of their H₂ production activity after 8 weeks of storage. When stored in argon at <5% humidity and room temperature, *R. palustris* coatings retained full H₂ production activity for 3 months, implicating oxidative damage as a key factor limiting coating storage. Overall, the results demonstrate that biocatalytic latex coatings are an attractive cell immobilization platform for preservation of bioactivity in the dry state.
Introduction

The encapsulation of living cells to create living hybrid materials for use as biocatalysts, biosensors, and bioremediation shows tremendous promise and has been the subject of extensive research for decades (Scott, 1987; Bjerketorp. et al., 2006; Wang. et al., 2010; Michelin and Roda, 2012). One of the fundamental challenges facing the successful commercialization of immobilized cell devices is how to preserve biological activity while retaining functionality and affordability for the end user (Bjerketorp. et al., 2006; Wang. et al., 2010; Michelin and Roda, 2012). Storage conditions that minimize metabolic activity, such as low temperatures or low relative humidity (i.e. freeze-dried, -80°C, under vacuum, etc.), are widely used and have a long history for microbial preservation, however some of these require continuous cold storage and very little attention has been paid to the application of non-refrigerated conditions for cell preservation in modern immobilization matrices such as sol-gels or latex (Bjerketorp. et al., 2006; Morgan. et al., 2006; Tessema. et al., 2006; Soltmann and Böttcher, 2008; Kuppardt. et al., 2009). Furthermore, almost all immobilization matrices require liquid immersion or a humid atmosphere in order to maintain bioactivity of the immobilized cells (Bjerketorp. et al., 2006; Michelin and Roda, 2012; Pannier. et al., 2012). A notable exception has been the development of freeze-gelation techniques of biologically active biocers (biological ceramic composites) (Koch. et al., 2007; Soltmann and Böttcher, 2008; Pannier. et al., 2012).

Adhesive latex binders are a stable, nontoxic, nanoporous matrix that have advantages compared to other immobilization matrices, such as alginate and sol-gel, because it is adhesive, economical (produced in very large quantities at low cost for the water borne coating industry), does not collapse upon drying, and can be used to immobilize very high concentrations of cells.
Experimental biocatalytic latex coatings have been designed for a variety of applications including: mercury detection, microbial fuel cell technology, high intensity chiral oxidations, and biocatalysis by thermophiles (Lyngberg, et al., 1999; Lyngberg, et al., 2005; Fidaleo, et al., 2006; Srikanth, et al., 2008). Adhesive latex-based coatings are also ideal for photosynthetic $H_2$ production because they provide a high surface area to volume ratio for incident light, efficient rates of gas diffusion, and can significantly increase cell longevity by protecting the microorganisms from mechanical degradation and contamination (Lyngberg, et al., 2001; Flickinger, et al., 2007). Indeed, latex coatings have been successfully developed for $H_2$ production using the purple non-sulfur phototroph, *Rhodopseudomonas palustris*, with stable rates of $H_2$ production ($2.08 \pm 0.01$ mmoles $H_2 \text{ m}^{-2} \text{ day}^{-1}$) observed for $>4000$ hours when cell/latex coatings are periodically hydrated in fresh liquid medium (Gosse, et al., 2007; Gosse, et al., 2010).

Preservation methods to ensure that latex-embedded cells can undergo long-term storage while maintaining activity have not been investigated. Coating preparation involves a controlled coalescence/drying step and, if needed, a subsequent short-term storage period, both affected by temperature and usually carried out at an elevated relative humidity of 60% relative humidity (Gosse, et al., 2007). While this strategy has proven successful when coatings are rehydrated for use within several days of preparation, for industrial “off-the-shelf” applications of microbial coatings, economics and practicality necessitates that coatings be stored in a dry state with stable reactivity over a much longer time frame (months-years). Storage conditions (temperature, humidity, $O_2$ tension, illumination or dark coating, etc.) must be defined to enable long-term dry storage of latex-embedded microbial cells preferably without the requirement for refrigeration.
In order to advance the development of latex-embedded *R. palustris* cells as lightweight, portable catalysts for H₂ production, we examined the effectiveness of 1) osmotic stabilizers such as glycerol, sucrose, trehalose, and sorbitol and 2) modifications to temperature, humidity and atmospheric O₂ concentration during the critical film formation coalescence and storage periods to extend the shelf life of *R. palustris* coatings.

**Results**

*Effect of osmotic stabilizers on H₂ production by R. palustris latex coatings*

Cells in coatings are subjected to desiccation stress, including osmotic shock, as the latex emulsion dries forming particle-particle coalescence and adhesion to the substrate resulting in cell immobilization. To moderate this stress, cell/latex formulations have been supplemented with sucrose, glycerol and other osmotic stabilizers (Yoo and Lee, 1993; Leslie, *et al.*, 1995; Lyngberg, *et al.*, 2001). To evaluate the utility of several osmotic stabilizers for maintaining H₂ production in *R. palustris* coatings following the initial dehydration period, latex formulations were supplemented with either sucrose, sorbitol, or trehalose, with or without glycerol, and stored for 2 days at 22 °C with 60% humidity. Regardless of the osmolyte combination tested, coatings of *R. palustris* maintained H₂ production for at least 85 days and after multiple media washes (Fig. 1a). As observed previously (Gosse, *et al.*, 2007; Gosse, *et al.*, 2010), H₂ production rates were highest immediately after hydration, and lower, but stable (0.82 mmol H₂ m⁻² h⁻¹ over ~80 days), after subsequent medium replacement/headsapce flushing events (Fig. 1b inset). Rates of H₂ production were similar among the stabilizer formulations tested (Fig. 1b).

*Coalescence and storage temperature of R. palustris coatings and H₂ production*
Here, we tested the hypothesis that coatings coalesced and stored at cold temperatures would maintain higher \(H_2\) production levels following hydration. \textit{R. palustris} latex coatings stabilized with glycerol and sucrose, sorbitol, or trehalose were dried and stored at either 22 °C or 4 °C (7 days, dark, 60% humidity). Initially (up to 5 days post-hydration), \(H_2\) production was much higher (6-10 fold) in coatings prepared and stored at 4 °C than those treated at 22 °C (Fig. 2a). However, \(H_2\) accumulation was similar between all treatments following 19 days of incubation (Fig. 2b), and 3 days after a subsequent medium replacement/flushing event (Fig. 2c).

\textit{H\(_2\) production after long-term storage by \textit{R. palustris} coatings}

To assess the ability of latex-embedded \textit{R. palustris} cells to maintain activity over long-term storage, coatings containing different osmotic stabilizers were stored up to 4 weeks at 60% humidity then assayed for \(H_2\) production (Table 1). Coatings stored for 14 days exhibited comparable \(H_2\) yields to fresh coatings that were rehydrated and assayed < 24 h after coalescence. After 28 days of storage, 2 of 3 sucrose coatings, 1 of 3 trehalose coatings, and all three sorbitol coatings failed to produce \(H_2\). The single active sucrose coating exhibited decreased \(H_2\) production yields (9.1% headspace \(H_2\)) compared to fresh coatings (29% \(H_2\) headspace), whereas the two active trehalose coatings exhibited modest losses of \(H_2\) production activity compared to fresh coatings (25% and 23% \(H_2\) headspace). None of the coatings generated \(H_2\) after 56 days of storage at 60% humidity.

To determine if \textit{R. palustris} coatings could maintain greater \(H_2\) production capability when stored under conditions of low relative humidity, coatings stabilized with glycerol and either sucrose, trehalose, or sorbitol were stored for 28 days at <5% or 60% relative humidity (Table 1). Sucrose and trehalose coatings retained 67% and 59% of their respective \(H_2\)
production activity when stored at <5% humidity over this time period. Importantly, all of the
sucrose and trehalose coating replicates (3 of each) retained H$_2$ production activity when stored
at <5% humidity, and there was little strip to strip coating (technical replicate) variability (Table
1). Sorbitol coatings did not produce H$_2$ after 28 days of storage, either at 60% or <5% humidity.

Additional experiments were conducted, with sucrose and trehalose as stabilizers (+
glycerol), to more fully assess the effects of storage humidity on H$_2$ production by *R. palustris*
coatings. As in our previous experiment, coatings stored for 28 days or longer exhibited very
little or no H$_2$ production activity when stored at 60% humidity and 22 °C (Table 2). In contrast,
when stored at <5% humidity the sucrose and trehalose coatings retained significant activity,
even after 56 days of storage (27% and 53% of the original activity, respectively). When stored
at <5% humidity, each coating replicate (3 of each) exhibited activity and the strip to strip
variability was relatively small. It is also important to note that when stored at <5% humidity,
very little activity was lost from the coatings between 4 and 8 weeks of storage. Coatings
initially dried under an atmosphere of <5% relative humidity (48 h) then placed at 60% humidity
behaved similarly to coatings that were coalesced and stored at 60% humidity – with complete
(or nearly complete) loss of H$_2$ production activity after 28 days of storage (Table 2).

*Respiratory activity of latex-embedded R. palustris after long-term storage.*

*R. palustris* cells embedded in latex were examined for anaerobic respiratory activity
after various storage periods using 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC). *R. palustris*
coatings stabilized with sucrose (+ glycerol) that had been stored in the dark for 2, 14, 28, or 56
days under either < 5% humidity or 60% humidity at 22 °C were rehydrated anaerobically in
PM(NF) medium and stained with CTC. CTC-stained coatings were examined with a confocal
laser-scanning microscope to evaluate cell anaerobic respiratory activity of the embedded *R. palustris* cells (Fig. 3). Image analysis revealed dense, actively respiring cells through the z-plane when the coatings were stored at < 5% humidity for 2 days, while a slightly thinner and less dense population was observed in coatings stored at 60% humidity (1.3 - 3.4 fold less CTC-stained cells based on image analysis). Longer storage times resulted in a marked decrease in cell respiratory activity, which was strongly dependent on the storage humidity. Coatings stored at 60% humidity contained very few CTC-stained cells after 14 days of storage, whereas coatings stored at < 5% humidity still contained an appreciable number of active cells (on average, ~ 15 fold more than the coatings stored at 60% humidity). CTC-stained cells were not detected in coatings stored at 60% humidity after 56 days storage, and <1% of the number of CTC-stained cells enumerated in the 2 day-old coatings remained in the coatings stored at <5% humidity.

Interestingly, the majority of CTC-stained cells observed in the 2 day-old coatings were located in the upper portion of the coating (top 10 µm), but after long-term storage, the CTC-stained cells clustered further into the coating interior. These results led us to hypothesize that cellular damage incurred in coatings over long-term storage periods was related to oxygen exposure. Thus, sucrose or trehalose (+glycerol) coatings were stored under argon for a period of 8-12 weeks at room temperature, and then assayed for H$_2$ production and CTC response. After 8 weeks of storage at <5% humidity, *R. palustris* coatings stored in an argon atmosphere exhibited 2 - 4 fold higher H$_2$ production activity than coatings stored in air (Table 3). After 12 weeks of storage H$_2$ accumulation was markedly higher (7 - 17 fold) in coatings stored in argon versus air. Notably, the H$_2$ production activity measured in *R. palustris* coatings that had been stored in argon for 12 weeks was equal to or greater than the activity observed in freshly prepared coatings (Tables 1, 2, 3). In accordance with these results, an abundance of CTC-stained cells were
observed in coatings stored at <5% humidity in argon over an 8 – 12 week period (Fig. 4).

Additionally, the CTC-stained cells did not cluster in the interior of the coating after storage in argon. These results demonstrate that H2 producing coatings of *R. palustris* can be stored dry, at room temperature, and maintain full activity for up to three months.

**Discussion**

An important technical hurdle that must be addressed before biocatalytic latex coatings can be used as “off-the-shelf” catalysts for H2 production, or other applications, is that of the stability of bioactivity as a function of long-term storage. In this study, latex-embedded cells of *R. palustris* were stored in a dry state at room temperature for up to 3 months while maintaining their original H2 production activity. Successful preservation of cell activity required the addition of select osmotic stabilizers, i.e. sucrose or trehalose, to the coating mixture, low relative humidity (<5%) and anoxic conditions during storage. It is important to note that we did not determine the dry storage lifetime of *R. palustris* coatings in this study. However, based on the observation that H2 production activity remained relatively stable over the final two sampling periods of this study (8 and 12 weeks), it seems likely that activity could be preserved in *R. palustris* coatings for greater periods of time. Overall, this study demonstrates that biolatex coatings have great potential as an “off-the-shelf” catalyst, considering that, with very little effort towards optimization, consistent retention of *R. palustris* activity was achieved following dry storage of coatings for at least 3 months at room temperature.

*R. palustris* coatings stored at 4 °C produced greater amounts of H2 than those stored at 22 °C in the days immediately following hydration. The primary goal of this particular experiment was to determine if decreased temperatures during the film formation and polymer
particle coalescence process and initial dry storage period would have an impact on H₂ production activity following hydration of the coatings – not to determine if low temperatures could be used as a long-term storage strategy. In our view, storage at room temperature is more compatible with many of the shipping, storage and handling constraints that would make latex embedded cells attractive as a lightweight, off-the-shelf biocatalyst or biosensor technology. The observation that *R. palustris* coatings exhibit a shorter lag in hydrogen production response time when prepared and stored at 4 °C has implications for applications, such as biosensors, where a ready-to-use system exhibiting immediate activity is essential.

Over time (10-20 days post-hydration), however, H₂ production activity was similar between *R. palustris* coatings that had been stored at 4 °C and 22 °C. These results could signify that coatings stored at 4 °C are capable of initiating H₂ production activity quicker than those stored at 22 °C, but that latex-embedded cells stored at 22 °C can eventually recover full activity after periods of short term (1 week) storage. Alternatively, diminished particle coalescence at 4 °C could lead to greater rates of acetate diffusion through the latex matrix upon hydration, thus greater H₂ yields for *R. palustris* coatings prepared and stored at this temperature. As the coatings age under hydration, the sugars tend to leach into the medium and particle coalescence resumes, thus the permeability decreases and acetate accessibility would become more uniform for both treatment temperatures (Lyngberg *et al.*, 2001).

Freeze-drying and controlled drying without freezing are the methods of choice used by industry to preserve microbial cells, and while these techniques are primarily applied to cell suspensions or pastes, they have also been investigated as techniques to preserve immobilized cells. For example, bacterial sensor cells targeting molecules as diverse as N-acylhomoserine or arsenite/arsenate have been air dried onto filter paper, lyophilized, and stored at 4 °C for 3.
months or 30 °C for 2 months without appreciable loss of reporter activity (Stocker, et al., 2003; Struss, et al., 2010). Freeze drying has also been applied to cells immobilized in sol-gels (Tessema, et al., 2006; Meunier, et al., 2010). However, freeze-dried cells must not be exposed to moisture and, despite high initial cell suspensions (greater than 10^8), survival rates of the original cell population can be as low as 0.1% (Bozoglu, et al., 1987; Miyamoto-Shinohara, et al., 2000; Miyamoto-Shinohara, et al., 2008). These cell viability rates may be acceptable for propagation of the strain but are incompatible with biocatalytic latex coatings that are engineered for a high reactivity per unit of surface area (high intensity) and where cell growth is limited. Nonetheless, because our results demonstrate that latex-embedded cells of *R. palustris* retain considerable activity when stored under low relative humidity and respond quicker when prepared at lower temperatures, the applicability of freeze drying biocatalytic latex coatings for long-term storage (> 1 year) should be evaluated (latex embedded *R. palustris* cells were previously shown to maintain activity after storage for 1 year at -80 °C (Gosse, et al., 2010)).

Regarding osmotic stabilizers, rates of H2 production by *R. palustris* coatings were quite similar for each of the formulations tested (sucrose, sorbitol, and trehalose ± glycerol) when the strips were fresh or stored for ≤ 2 weeks. Coatings prepared with either trehalose or sucrose retained 31-67% of their H2 production activity through 28 days under low humidity and 27-53% activity through 56 days of storage. In contrast, sorbitol-stabilized coatings were inactive regardless of the relative humidity levels beyond 2 weeks storage time. Although we did not investigate the underlying factors responsible for the differences in stabilizer performance, other studies have concluded that the efficacy of sorbitol as an osmoprotectant is quite variable (de Valdez, et al., 1983; Carvalho, et al., 2003), resulting in a greater emphasis on sucrose and trehalose as stabilizers (Leslie, et al., 1995; Lyngberg, et al., 2001).
The two elements found to be critical for preserving H₂ production activity in *R. palustris* coatings were 1) low relative humidity and 2) low O₂ levels during the storage period. Since slower drying rates allow for greater polymer particle mobility, coatings that are dried at 60% humidity have greater permeability than those dried at lower humidity (Lyngberg, *et al.*, 1999) resulting in greater porosity for gas and nutrient diffusion (Sperry, *et al.*, 1994; Ma, *et al.*, 2005).

As metabolism progresses, cells accumulate extracellular metabolites which, in high concentrations, may become either toxic or increase osmotic pressure. Storage at 60% humidity may provide the latex-embedded cells enough moisture to support low levels of metabolic activity, which could result in energy depletion over time or accumulation of toxic metabolites in the pore space adjacent to cells. Visual inspection of the *R. palustris* coatings also revealed differences between coatings stored under low or high humidity (data not shown). The characteristic red-purplish pigmentation of *R. palustris* dulls to a light red/orange color over time during dry storage at 60% humidity, suggesting loss of light-harvesting bacteriochlorophyll or carotenoids. Coatings stored at <5% humidity were much more resilient to pigmentation loss.

Oxidative stress is well known as a cause of cell damage and death during long-term storage (Dimmick, *et al.*, 1961; Meng, *et al.*, 2008). Oxidative damage to DNA, proteins, and particularly the cell membrane, has been implicated as a major contributor to the viability losses often observed when dried microorganisms are exposed to air for extended periods (Marshall, *et al.*, 1974; Israeli, *et al.*, 1975; Teixeira, *et al.*, 1996; Vriezen, *et al.*, 2007; Scherber, *et al.*, 2009).

Water-deficient cells are unable to actively neutralize or excrete oxygen radicals or repair oxidative damage, thus cellular injury would inevitably and slowly accumulate until a threshold is reached beyond which cell recovery is improbable. In this study, we provide two lines of evidence that oxidative stress is an impediment to the long-term storage of *R. palustris* coatings.
First, coatings stored under an argon atmosphere retained >10 times greater \( \text{H}_2 \) production activity than those stored under air, even under conditions of low relative humidity where metabolic activity should be minimal. Second, as storage time elapsed under air, respiratory activity (assayed under anaerobic conditions) in rehydrated coatings was detected in \textit{R. palustris} cells tended that clustered towards the interior of the latex coating (Fig. 4) — where \( \text{O}_2 \) exposure during storage would be less than at the edges of the coating. In contrast, active cells were detected closer to the surface of rehydrated coatings after stored under argon (Fig. 4).

The enzyme responsible for \( \text{H}_2 \) production in \textit{R. palustris}, nitrogenase, is highly sensitive to oxygen (Gallon, 1992); therefore, exposure to \( \text{O}_2 \) during long-term storage could result in longer lag times associated with \( \text{H}_2 \) production (time required for repair or de novo synthesis of nitrogenase). Indeed, longer lag periods were noted for \textit{R. palustris} coatings stored under air versus argon (data not shown), which could account for the large difference in \( \text{H}_2 \) yields exhibited by coatings stored under these two conditions immediately following hydration (Table 3). Because coatings stored under argon continued to produce \( \text{H}_2 \) at rates > 10x that of air-stored coatings up to 40 days after rehydration and after medium replacement, it is probable that nitrogenase inactivation is not the only damage incurred by latex-embedded \textit{R. palustris} cells upon long-term storage in the presence of air. Accordingly, the CTC staining experiments revealed that general cell respiratory activity was compromised to a much greater extent in latex-embedded \textit{R. palustris} stored in air.

This study provides a method by which biocatalytic latex coatings of \textit{R. palustris} can be stored in a dry state at room temperature for up to 12 weeks and retain biohydrogen production activity. To our knowledge, this is the only study of immobilization and storage techniques that show the recovery of photobiological activity by embedded cells after desiccation and storage at
room temperature over an extended period of time. We believe the long-term storage properties of biocatalytic latex coatings make it an attractive technology for a myriad of applications. For example, cell immobilization technologies applied to bioconversion, alternative fuel production, bioremediation, solar energy trapping, and food processing could benefit from long-term storage at room temperature using the methods described herein (Junter and Jouenne, 2004).

**Experimental procedures**

*Bacterial strain, media, growth conditions, and latex characteristics*

*Rhodopseudomonas palustris* CGA009 was kindly provided by Dr. Caroline Harwood, University of Washington. This strain produces hydrogen via three isozymes of the nitrogenase protein in an anaerobic environment at higher yields than the wild type due to an inactive uptake hydrogenase caused by a spontaneous frameshift mutation in the hydrogen sensor protein, *hupV* (Oda, *et al.*, 2005; Rey, *et al.*, 2006). *R. palustris* was cultured anaerobically in nitrogen fixing photosynthetic medium, PM(NF), supplemented with 20 mM acetate (unless otherwise noted, PM(NF) used throughout this study contained 20 mM acetate) in sealed glass serum bottles under a N₂ atmosphere (Gosse, *et al.*, 2007). *R. palustris* was incubated statically under constant illumination at 60 µE with 60 W incandescent light bulbs at 31 °C.

Latecies KAK4391 and Rhoplex™ SF012 (Rohm and Hass Co., Philadelphia, PA) latex formulations, both adjusted to pH 7.0, were used for this study. Latex KAK3941 is a vinyl acetate-co-acrylate that does not include biocide or hydroxyethylcellulose surface grafting, has a glass transition temperature (T_g) of 8.1 °C, an average particle size of 280 nm, and a percent solids of 52.5%. Rhoplex SF012 is a commercially available, acrylic co-polymer binder without biocide containing a solids content of 43.5%.
Preparation of *R. palustris* latex coatings (Supp. Fig. 1)

*R. palustris* cells were harvested in early stationary phase (OD$_{660}$ ∼0.8) by centrifugation at 7,600 x $g$ for 15 min at room temperature. Cell pellets were suspended in 50 ml PM(NF) medium without acetate and transferred to pre-weighed 50 mL Falcon tubes. After centrifugation (as above), the supernatant was removed and the wet weight of the cell pellet was determined. Prior to latex addition, the bacterial cell paste was first mixed with the indicated amount of glycerol and/or sucrose, sorbitol, or trehalose. The latex emulsions for coatings were prepared based on the formulation ratio of 1.2 g wet cell weight, 350 µl of 1.7 M sucrose, sorbitol, or trehalose, 150 µl of 100% glycerol (exceptions are noted), and 1 ml of latex. The two initial experiments (Figs. 1 and 2) were conducted using KAK4391 latex; however, due to a discontinuation of this product by the manufacturer, the remaining studies were performed with Rhoplex emulsion SF012. Importantly, when using the SF012 latex, the polyester template was first cleansed with a small amount of 1 M HCl to minimize hydrophobic tension in the formulation upon spreading.

*R. palustris* latex coatings were prepared as strips using a template design consisting of a glass support, a 125-µm thick polyester sheet (DuPont Melinex 454, Tekra Corp, NJ), and an adhesive vinyl mask (84 µm thick, Con-Tact, Stamford, CT). The polyester sheet was pre-cut with parallel lines separated by 1 cm to define the width of each strip, and attached to a glass support covered with double-sided Scotch tape (strips perpendicular to the tape). An adhesive vinyl mask with a pre-cut rectangle (5 cm long to define the length of each strip; modified to 3.5 cm) was placed on top of the polyester so that its parallel pre-cut lines were in the center of the
mask opening. The width of the mask opening was determined by the number of polyester strips plus an additional 0.5 cm on each end (for template design details (Gosse and Flickinger, 2010)).

Coatings were prepared under aerobic conditions in an acrylic glove box (Plas By Labs, Lansing, MI) at 22 °C and 60% humidity, unless otherwise noted. Humidity was measured using a dew point, wet-bulb humidity thermometer (Fisher Scientific, Pittsburgh PA). The latex/cell formulation was transferred from a Falcon tube onto an assembled template mask where it was then spread across the top of the mask along the width of the polyester window with a pipette, minimizing bubble formation (see Supporting Figure 1 in supplemental information). A 26-wire wound Mayer rod (Paul N. Gardner Co., Pompano Beach, FL) was drawn by hand down the template mask in order to spread the formulation. The coatings were allowed to dry for 24 h (unless otherwise noted) in the glove box before removing the mask. Each individual polyester strip (1 x 5 cm, unless otherwise noted) “painted” with embedded cells was then removed from the glass support and hydrated with 10 mL PM(NF) medium in Balch tubes (Bellco Biotechnology Inc., Vineland, NJ) (Gosse, et al., 2007). Tubes were sealed with butyl septum stoppers, and flushed with argon for 30 min to produce an anaerobic environment for H₂ production (16.5 mL headspace). *R. palustris* coatings in sealed Balch tubes were incubated statically under a light intensity of 60 μE at 31 °C.

Preparation of latex coatings under modified storage conditions

Coatings were prepared from a single culture of *R. palustris* using a separate template mask for each stabilizer combination. After the latex/cell/stabilizer mixture was allowed to coalesce for 24 h, triplicate coatings from each treatment were removed from their respective masks and placed into separate Balch tubes containing PM(NF) medium to assay H₂ production
(argon atmosphere; H₂ accumulation was measured at 5 day intervals over 20 days). The remaining coatings were removed from the mask, placed in Petri dishes, covered with foil, and stored at 22 °C under 60% humidity for 14, 28, or 56 days before assaying for H₂ production. One set of triplicate coatings was stored at < 5% humidity for 28 days.

Gas analysis

Headspace gas analysis was performed using an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a thermal conductivity detector and a HP-Molseive column (30 m × 0.32 mm × 25 uM). Argon was the carrier gas and the oven, detector, and inlet temperatures settings were 50 °C, 275 °C, and 105 °C, respectively, yielding a RT of 6.02 min for H₂ and baseline separation of N₂ and O₂. H₂ was quantified by comparison of peak areas to standard curves constructed from known amounts of H₂ gas.

CTC staining and confocal microscopy

The respiratory activity of R. palustris CGA009 cells in latex coatings was assessed under anaerobic conditions using 5-cyano-2,3-ditolyl tetrazolium chloride dye (CTC; Sigma-Aldrich, St. Louis, MO). CTC is internalized and reduced by actively respiring cells to a fluorescent CTC-formazan that can be detected by epifluorescence microscopy (Rodriguez, et al., 1992; Yu and McFeters, 1994).

Coatings of R. palustris CGA009, prepared with a latex/sucrose/glycerol formulation, were dried at 60% humidity for 3 h and then stored in the dark at either 60% or < 5% humidity for 2, 14, 28, or 56 days. Additionally, coatings, prepared with sucrose or trehalose, were stored in septa-sealed Balch tubes containing drierite for 8 – 12 weeks under aerobic (a needle attached
to a 0.2 micron syringe filter was passed through the septum) or anaerobic conditions (tubes were sealed and flushed extensively with argon; low O₂ concentrations were confirmed with by GC-TCD). Coatings were hydrated in PM(NF) medium and pre-incubated in sealed Balch tubes under an argon headspace for 4 days, after which CTC dye was injected into the sealed Balch tubes to a final concentration of 4 mM. Coatings were incubated anaerobically with CTC in the dark for 1 h with constant shaking (100 rpm) at 31 °C. Heat-treated (85 °C for 0.5 h) and unstained latex coatings were also examined as controls.

A confocal laser-scanning microscope (LSM 510 Meta, Carl Zeiss Microimaging, Inc.) equipped with a HeNe laser was used to view the CTC-stained latex coatings. Images were collected at an excitation wavelength of 543 nm, a master gain of 633 V, and with an alpha Plan-Fluor 100X/1.45 oil objective. The confocal microscope facilitated viewing the cells at different depths (z-axis) within the latex coating matrix. Multiple images were analyzed and fluorescent cells were enumerated using the AlphaEase FC counting software (Alphalmager 3400; Alpha Innotech Corporation, San Leandro, CA).

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References


**Figure Legends**

**Figure 1.** *H₂* production by *R. palustris* latex coatings containing different osmotic stabilizers (sucrose, sorbitol, or trehalose; with or without glycerol). Freshly prepared *R. palustris* coatings were stored at 22 °C under 60% humidity for 2 days prior to hydration in PM(NF) medium and initiation of the *H₂* production assays. (A) *H₂* production is presented as percent accumulation in the headspace over time where the headspace of each is tube is refreshed periodically with argon upon medium replacement (~ every 2 weeks). (B) Cumulative *H₂* production from the same coatings with arrows representing each medium replacement/headspace-flushing event. The inset in (B) shows the average rate of *H₂* production of all coatings, 0.82 mmoles *H₂* m⁻² h⁻¹. Symbols represent average values obtained from 2 coatings.
Figure 2. Hydrogen production by *R. palustris* cells embedded in latex containing glycerol along with sucrose, sorbitol, or trehalose and prepared/stored at 4 °C (gray bars) or 22 °C (striped bars) under 60% humidity for 7 days. Hydrogen production is presented as the percent H$_2$ in the headspace (A) 5 days post-hydration, (B) 19 days post-hydration, and (C), and 3 days after replacing the medium and flushing the headspace on Day 19. Bars are average values of duplicate coatings and stars delimit the range of values.

Figure 3. Three-dimensional views of *R. palustris* coatings (+ sucrose/glycerol) stained with CTC (Z and X axes are 13 and 125 µM, respectively). Coatings were stored at room temperature in the dark for 2, 14, 28 or 56 days at <5% humidity (A-D) or 60% humidity (E-H).

Figure 4. Three-dimensional views of *R. palustris* coatings ((+ sucrose/glycerol) stained with CTC (Z and X axes are 9 and 125 µM, respectively). Coatings were stored at room temperature in the dark at <5% humidity under argon for 56 or 84 days (A, B) or air for 56 or 84 days (C, D).
Table 1. Hydrogen production by *R. palustris* coatings after storage for up to 56 days at 22 °C and 60% humidity.

<table>
<thead>
<tr>
<th>Storage period (days)</th>
<th>H$_2$ accumulation (% headspace)$^a$</th>
<th>Sucrose$^b$</th>
<th>Trehalose$^b$</th>
<th>Sorbitol$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1</td>
<td>29.0 ± 0.5$^d$</td>
<td>30.4 ± 6.4</td>
<td>31.9 ± 8.9</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>25.2 ± 1.9</td>
<td>21.3 ± 0.4</td>
<td>32.2 ± 12.6</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>3.3 ± 5.6</td>
<td>16.8 ± 14.5</td>
<td>0 ± 0</td>
<td></td>
</tr>
<tr>
<td>28$^c$</td>
<td>19.3 ± 1.7</td>
<td>17.9 ± 0.4</td>
<td>0 ± 0</td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td></td>
</tr>
</tbody>
</table>

$^a$H$_2$ accumulation was measured 20 days after coatings had been rehydrated in PM(NF) medium in closed vessels containing an argon atmosphere.

$^b$Coatings contained glycerol plus the indicated osmolyte stabilizers

$^c$Coatings were stored under <5% relative humidity; all others were stored at 60% humidity

$^d$Values are averages from 3 replicate strips ± SD
Table 2. Hydrogen production by *R. palustris* coatings after storage for up to 56 days at 22 °C and <5% or 60% humidity.

<table>
<thead>
<tr>
<th>Storage period (days)</th>
<th>H₂ accumulation (% headspace)&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sucrose&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;5%</td>
<td>60%</td>
<td>60%&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Trehalose&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>7</td>
<td>51.1 ± 5.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9.4 ± 0.9</td>
<td>9.0 ± 2.4</td>
<td></td>
<td>35.2 ± 1.0</td>
<td>14.1 ± 3.4</td>
</tr>
<tr>
<td>28</td>
<td>15.7 ± 0.7</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td></td>
<td>20.2 ± 0.8</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>56</td>
<td>13.8 ± 4.4</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td></td>
<td>18.5 ± 0.6</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

<sup>a</sup>H₂ accumulation in argon measured 20 days post rehydration in PM(NF)

<sup>b</sup>Coatings contained glycerol plus the indicated osmolyte stabilizer

<sup>c</sup>Coatings dried for 48 hours at <5% humidity, then stored at 60% humidity

<sup>d</sup>Values are averages from 3 replicate strips ± SD
Table 3. Hydrogen production by *R. palustris* coatings after storage for 8 – 12 weeks at <5% relative humidity under air or argon.

| Storage period (weeks) | H₂ accumulation (% headspace)
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Sucrose (+glycerol)</td>
</tr>
<tr>
<td></td>
<td>Air</td>
</tr>
<tr>
<td>8</td>
<td>12.7 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>8 (2&lt;sup&gt;nd&lt;/sup&gt; flush)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.5 ± 2.3</td>
</tr>
<tr>
<td>12</td>
<td>9.5 ± 1.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>H₂ accumulation in argon 10 days post rehydration in PM(NF) medium

<sup>b</sup>H₂ accumulation in argon (10 day incubation) following the initial H₂ production period (20 days) and one flushing/medium refresh event

<sup>c</sup>Values are averages from 3 replicate strips ± SD
**Supporting Information**

**Supporting Figure 1.** Illustration of the *R. palustris* latex coating method. (A) Cultures of *R. palustris* grown anaerobically in serum vials; (B) Pellet of *R. palustris* cells from the 4 serum vials; (C) Application of cell/latex/osmotic stabilizer mixture across the top of a pre-cut polyester mask in a humidity-controlled chamber; (D) Spreading cell/latex mixture using a Mayer rod; (E) Drying coatings in the pre-cut mask under air with humidity control; (F) Coating strips hydrated in PM(NF) medium under argon headspace in Balch tubes.
Fig. 1

a

\[ y = 0.0096x + 0.0896 \]

\[ R^2 = 0.9946 \]

b

\[ H_2 \text{ (mmoles)} \]

\[ 0 \quad 0.4 \quad 0.8 \quad 1.2 \]

\[ 0 \quad 15 \quad 30 \quad 45 \quad 60 \quad 75 \quad 90 \]

- Sucrose (- glycerol)
- Sorbitol (- glycerol)
- Trehalose (- glycerol)
- Sucrose (+ glycerol)
- Sorbitol (+ glycerol)
- Trehalose (+ glycerol)
Fig. 3

<5% humidity

A  2 d
B  14 d
C  28 d
D  56 d

60% humidity

E  2 d
F  14 d
G  28 d
H  56 d
Fig. 4

Argon

Air

a  56 d
b  84 d
c  56 d
d  84 d
Supp. Fig. 1