Preservation of H_2 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 O_2 on preservation of H_2 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 H_2 production. Beyond 2 weeks of storage, sorbitol-treated coatings lost all H_2 production activity, whereas considerable H_2 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 H_2 production. After 4 weeks storage under air at 60% humidity, coatings produced only trace amounts of H_2 (0-0.1% headspace accumulation), whereas those stored at <5% humidity retained 27-53% of their H_2 production activity after 8 weeks of storage. When stored in argon at <5% humidity and room temperature, *R. palustris* coatings retained full H_2 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; Michelini 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; Michelini 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; Michelini 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

(Flickinger, *et al.*, 2007). 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 ± 0.01 mmoles H_2 m^{-2} 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 H_2 production, we examined the effectiveness of 1) osmotic stabilizers such as glycerol, sucrose, trehalose, and sorbitol and 2) modifications to temperature, humidity and atmospheric O_2 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 H_2 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 H_2 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 H_2 production for at least 85 days and after multiple media washes (Fig. 1a). As observed previously (Gosse, *et al.*, 2007; Gosse, *et al.*, 2010), H_2 production rates were highest immediately after hydration, and lower, but stable (0.82 mmol H_2 m^{-2} h^{-1} over ~80 days), after subsequent medium replacement/headspace flushing events (Fig. 1b inset). Rates of H_2 production were similar among the stabilizer formulations tested (Fig. 1b).

Coalescence and storage temperature of R. palustris coatings and H_2 production

Here, we tested the hypothesis that coatings coalesced and stored at cold temperatures would maintain higher H_2 production levels following hydration. *R. palustris* latex coatings stabilized with glycerol and sucrose, sorbitol, or trehalose were dried and stored at either 22 ºC or 4 ºC (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 ºC than those treated at 22 ºC (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).

H_2 production after long-term storage by R. palustris coatings

To assess the ability of latex-embedded *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 sorbital 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 *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

153 laser-scanning microscope to evaluate cell anaerobic respiratory activity of the embedded *R.*
154 *palustris* cells (Fig. 3). Image analysis revealed dense, actively respiring cells through the z-
155 plane when the coatings were stored at < 5% humidity for 2 days, while a slightly thinner and
156 less dense population was observed in coatings stored at 60% humidity (1.3 – 3.4 fold less CTC-
157 stained cells based on image analysis). Longer storage times resulted in a marked decrease in cell
158 respiratory activity, which was strongly dependent on the storage humidity. Coatings stored at
159 60% humidity contained very few CTC-stained cells after 14 days of storage, whereas coatings
160 stored at < 5% humidity still contained an appreciable number of active cells (on average, ~ 15
161 fold more than the coatings stored at 60% humidity). CTC-stained cells were not detected in
162 coatings stored at 60% humidity after 56 days storage, and <1% of the number of CTC-stained
163 cells enumerated in the 2 day-old coatings remained in the coatings stored at <5% humidity.
164 Interestingly, the majority of CTC-stained cells observed in the 2 day-old coatings were
165 located in the upper portion of the coating (top 10 μm), but after long-term storage, the CTC-
166 stained cells clustered further into the coating interior. These results led us to hypothesize that
167 cellular damage incurred in coatings over long-term storage periods was related to oxygen
168 exposure. Thus, sucrose or trehalose (+glycerol) coatings were stored under argon for a period of
169 8-12 weeks at room temperature, and then assayed for H_2 production and CTC response. After 8
170 weeks of storage at <5% humidity, *R. palustris* coatings stored in an argon atmosphere exhibited
171 2 – 4 fold higher H_2 production activity than coatings stored in air (Table 3). After 12 weeks of
172 storage H_2 accumulation was markedly higher (7 – 17 fold) in coatings stored in argon versus air.
173 Notably, the H_2 production activity measured in *R. palustris* coatings that had been stored in
174 argon for 12 weeks was equal to or greater than the activity observed in freshly prepared coatings
175 (Tables 1, 2, 3). In accordance with these results, an abundance of CTC-stained cells were

176 observed in coatings stored at <5% humidity in argon over an 8 – 12 week period (Fig. 4).
177 Additionally, the CTC-stained cells did not cluster in the interior of the coating after storage in
178 argon. These results demonstrate that H_2 producing coatings of *R. palustris* can be stored dry, at
179 room temperature, and maintain full activity for up to three months.
180
181 **Discussion**
182 An important technical hurdle that must be addressed before biocatalytic latex coatings
183 can be used as "off-the-shelf" catalysts for H_2 production, or other applications, is that of the
184 stability of bioactivity as a function of long-term storage. In this study, latex-embedded cells of
185 *R. palustris* were stored in a dry state at room temperature for up to 3 months while maintaining
186 their original H_2 production activity. Successful preservation of cell activity required the addition
187 of select osmotic stabilizers, i.e. sucrose or trehalose, to the coating mixture, low relative
188 humidity (<5%) and anoxic conditions during storage. It is important to note that we did not
189 determine the dry storage lifetime of *R. palustris* coatings in this study. However, based on the
190 observation that H_2 production activity remained relatively stable over the final two sampling
191 periods of this study (8 and 12 weeks), it seems likely that activity could be preserved in *R.*
192 *palustris* coatings for greater periods of time. Overall, this study demonstrates that biolatex
193 coatings have great potential as an "off-the-shelf" catalyst, considering that, with very little effort
194 towards optimization, consistent retention of *R. palustris* activity was achieved following dry
195 storage of coatings for at least 3 months at room temperature.
196 *R. palustris* coatings stored at 4 °C produced greater amounts of H_2 than those stored at
197 22 °C in the days immediately following hydration. The primary goal of this particular
198 experiment was to determine if decreased temperatures during the film formation and polymer

199 particle coalescence process and initial dry storage period would have an impact on H_2
200 production activity following hydration of the coatings – not to determine if low temperatures
201 could be used as a long-term storage strategy. In our view, storage at room temperature is more
202 compatible with many of the shipping, storage and handling constraints that would make latex
203 embedded cells attractive as a lightweight, off-the-shelf biocatalyst or biosensor technology. The
204 observation that *R. palustris* coatings exhibit a shorter lag in hydrogen production response time
205 when prepared and stored at 4 °C has implications for applications, such as biosensors, where a
206 ready-to-use system exhibiting immediate activity is essential.
207 Over time (10-20 days post-hydration), however, H_2 production activity was similar
208 between *R. palustris* coatings that had been stored at 4 °C and 22 °C. These results could signify
209 that coatings stored at 4 °C are capable of initiating H_2 production activity quicker than those
210 stored at 22 °C, but that latex-embedded cells stored at 22 °C can eventually recover full activity
211 after periods of short term (1 week) storage. Alternatively, diminished particle coalescence at 4
212 °C could lead to greater rates of acetate diffusion through the latex matrix upon hydration, thus
213 greater H_2 yields for *R. palustris* coatings prepared and stored at this temperature. As the
214 coatings age under hydration, the sugars tend to leach into the medium and particle coalescence
215 resumes, thus the permeability decreases and acetate accessibility would become more uniform
216 for both treatment temperatures (Lyngberg, *et al.*, 2001).
217 Freeze-drying and controlled drying without freezing are the methods of choice used by
218 industry to preserve microbial cells, and while these techniques are primarily applied to cell
219 suspensions or pastes, they have also been investigated as techniques to preserve immobilized
220 cells. For example, bacterial sensor cells targeting molecules as diverse as N-acylhomoserine or
221 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 H_2 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 H_2 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).

245 The two elements found to be critical for preserving H_2 production activity in *R. palustris*
246 coatings were 1) low relative humidity and 2) low O_2 levels during the storage period. Since
247 slower drying rates allow for greater polymer particle mobility, coatings that are dried at 60%
248 humidity have greater permeability than those dried at lower humidity (Lyngberg, *et al.*, 1999)
249 resulting in greater porosity for gas and nutrient diffusion (Sperry, *et al.*, 1994; Ma, *et al.*, 2005).
250 As metabolism progresses, cells accumulate extracellular metabolites which, in high
251 concentrations, may become either toxic or increase osmotic pressure. Storage at 60% humidity
252 may provide the latex-embedded cells enough moisture to support low levels of metabolic
253 activity, which could result in energy depletion over time or accumulation of toxic metabolites in
254 the pore space adjacent to cells. Visual inspection of the *R. palustris* coatings also revealed
255 differences between coatings stored under low or high humidity (data not shown). The
256 characteristic red-purplish pigmentation of *R. palustris* dulls to a light red/orange color over time
257 during dry storage at 60% humidity, suggesting loss of light-harvesting bacteriochlorophyll or
258 carotenoids. Coatings stored at <5% humidity were much more resilient to pigmentation loss.
259 Oxidative stress is well known as a cause of cell damage and death during long-term
260 storage (Dimmick, *et al.*, 1961; Meng, *et al.*, 2008). Oxidative damage to DNA, proteins, and
261 particularly the cell membrane, has been implicated as a major contributor to the viability losses
262 often observed when dried microorganisms are exposed to air for extended periods (Marshall, *et*
263 *al.*, 1974; Israeli, *et al.*, 1975; Teixeira, *et al.*, 1996; Vriezen, *et al.*, 2007; Scherber, *et al.*, 2009).
264 Water-deficient cells are unable to actively neutralize or excrete oxygen radicals or repair
265 oxidative damage, thus cellular injury would inevitably and slowly accumulate until a threshold
266 is reached beyond which cell recovery is improbable. In this study, we provide two lines of
267 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 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 *R. palustris* cells tended that clustered towards the interior of the latex coating (Fig. 4) – where 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 H_2 production in *R. palustris*, nitrogenase, is highly sensitive to oxygen (Gallon, 1992); therefore, exposure to O_2 during long-term storage could result in longer lag times associated with H_2 production (time required for repair or de novo synthesis of nitrogenase). Indeed, longer lag periods were noted for *R. palustris* coatings stored under air versus argon (data not shown), which could account for the large difference in H_2 yields exhibited by coatings stored under these two conditions immediately following hydration (Table 3). Because coatings stored under argon continued to produce 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 *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 *R. palustris* stored in air.

This study provides a method by which biocatalytic latex coatings of *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

291 room temperature over an extended period of time. We believe the long-term storage properties
292 of biocatalytic latex coatings make it an attractive technology for a myriad of applications. For
293 example, cell immobilization technologies applied to bioconversion, alternative fuel production,
294 bioremediation, solar energy trapping, and food processing could benefit from long-term storage
295 at room temperature using the methods described herein (Junter and Jouenne, 2004).
296
297 **Experimental procedures**
298 *Bacterial strain, media, growth conditions, and latex characteristics*
299 *Rhodopseudomonas palustris* CGA009 was kindly provided by Dr. Caroline Harwood,
300 University of Washington. This strain produces hydrogen via three isozymes of the nitrogenase
301 protein in an anaerobic environment at higher yields than the wild type due to an inactive uptake
302 hydrogenase caused by a spontaneous frameshift mutation in the hydrogen sensor protein, *hupV*
303 (Oda, *et al.*, 2005; Rey, *et al.*, 2006). *R. palustris* was cultured anaerobically in nitrogen fixing
304 photosynthetic medium, PM(NF), supplemented with 20 mM acetate (unless otherwise noted,
305 PM(NF) used throughout this study contained 20 mM acetate) in sealed glass serum bottles
306 under a N_2 atmosphere (Gosse, *et al.*, 2007). *R. palustris* was incubated statically under constant
307 illumination at 60 μE with 60 W incandescent light bulbs at 31 °C.
308 Latecies KAK4391 and Rhoplex™ SF012 (Rohm and Hass Co., Philadelphia, PA) latex
309 formulations, both adjusted to pH 7.0, were used for this study. Latex KAK3941 is a vinyl
310 acetate-*co*-acrylate that does not include biocide or hydroxyethylcellulose surface grafting, has a
311 glass transition temperature (Tg) of 8.1 °C, an average particle size of 280 nm, and a percent
312 solids of 52.5%. Rhoplex SF012 is a commercially available, acrylic *co*-polymer binder without
313 biocide containing a solids content of 43.5%.

314

315 *Preparation of R. palustris latex coatings (Supp. Fig.1)*

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

329 *R. palustris* latex coatings were prepared as strips using a template design consisting of a
330 glass support, a 125-µm thick polyester sheet (DuPont Melinex 454, Tekra Corp, NJ), and an
331 adhesive vinyl mask (84 µm thick, Con-Tact, Stamford, CT). The polyester sheet was pre-cut
332 with parallel lines separated by 1 cm to define the width of each strip, and attached to a glass
333 support covered with double-sided Scotch tape (strips perpendicular to the tape). An adhesive
334 vinyl mask with a pre-cut rectangle (5 cm long to define the length of each strip; modified to 3.5
335 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 ºC 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 H_2 production (16.5 mL headspace). *R. palustris* coatings in sealed Balch tubes were incubated statically under a light intensity of 60 µE at 31 ºC.

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 H_2 production

359 (argon atmosphere; H_2 accumulation was measured at 5 day intervals over 20 days). The
360 remaining coatings were removed from the mask, placed in Petri dishes, covered with foil, and
361 stored at 22 °C under 60% humidity for 14, 28, or 56 days before assaying for H_2 production.
362 One set of triplicate coatings was stored at < 5% humidity for 28 days.
363
364 *Gas analysis*
365 Headspace gas analysis was performed using an Agilent 6890 gas chromatograph
366 (Agilent Technologies, Santa Clara, CA) equipped with a thermal conductivity detector and a
367 HP-Molseive column (30 m × 0.32 mm × 25 uM). Argon was the carrier gas and the oven,
368 detector, and inlet temperatures settings were 50 °C, 275 °C, and 105 °C, respectively, yielding a
369 RT of 6.02 min for H_2 and baseline separation of N_2 and O_2. H_2 was quantified by comparison of
370 peak areas to standard curves constructed from known amounts of H_2 gas.
371
372 *CTC staining and confocal microscopy*
373 The respiratory activity of *R. palustris* CGA009 cells in latex coatings was assessed
374 under anaerobic conditions using 5-cyano-2,3-ditolyl tetrazolium chloride dye (CTC; Sigma-
375 Aldrich, St. Louis, MO). CTC is internalized and reduced by actively respiring cells to a
376 fluorescent CTC-formazan that can be detected by epifluorescence microscopy (Rodriguez, *et*
377 *al.*, 1992; Yu and McFeters, 1994).
378 Coatings of *R. palustris* CGA009, prepared with a latex/sucrose/glycerol formulation,
379 were dried at 60% humidity for 3 h and then stored in the dark at either 60% or < 5% humidity
380 for 2, 14, 28, or 56 days. Additionally, coatings, prepared with sucrose or trehalose, were stored
381 in septa-sealed Balch tubes containing drierite for 8 – 12 weeks under aerobic (a needle attached

382 to a 0.2 micron syringe filter was passed through the septum) or anaerobic conditions (tubes were
383 sealed and flushed extensively with argon; low O_2 concentrations were confirmed with by GC-
384 TCD). Coatings were hydrated in PM(NF) medium and pre-incubated in sealed Balch tubes
385 under an argon headspace for 4 days, after which CTC dye was injected into the sealed Balch
386 tubes to a final concentration of 4 mM. Coatings were incubated anaerobically with CTC in the
387 dark for 1 h with constant shaking (100 rpm) at 31 °C. Heat-treated (85 °C for 0.5 h) and
388 unstained latex coatings were also examined as controls.

389 A confocal laser-scanning microscope (LSM 510 Meta, Carl Zeiss Microimaging, Inc.)
390 equipped with a HeNe1 laser was used to view the CTC-stained latex coatings. Images were
391 collected at an excitation wavelength of 543 nm, a master gain of 633 V, and with an alpha Plan-
392 Fluor 100X/1.45 oil objective. The confocal microscope facilitated viewing the cells at different
393 depths (z-axis) within the latex coating matrix. Multiple images were analyzed and fluorescent
394 cells were enumerated using the AlphaEase FC counting software (AlphaImager 3400; Alpha
395 Innotech Corporation, San Leandro, CA).

396

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403

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Figure Legends

Figure 1. H_2 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 H_2 production assays. (A) H_2 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 H_2 production from the same coatings with arrows representing each medium replacement/headspace-flushing event. The inset in (B) shows the average rate of H_2 production of all coatings, 0.82 mmoles H_2 m^{-2} h^{-1}. Symbols represent average values obtained from 2 coatings.

575

576 **Figure 2.** Hydrogen production by *R. palustris* cells embedded in latex containing glycerol along
577 with sucrose, sorbitol, or trehalose and prepared/stored at 4 °C (gray bars) or 22 °C (striped bars)
578 under 60% humidity for 7 days. Hydrogen production is presented as the percent H_2 in the
579 headspace (A) 5 days post-hydration, (B) 19 days post-hydration, and (C), and 3 days after
580 replacing the medium and flushing the headspace on Day 19. Bars are average values of
581 duplicate coatings and stars delimit the range of values.

582

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

586

587 **Figure 4.** Three-dimensional views of *R. palustris* coatings ((+ sucrose/glycerol) stained with
588 CTC (Z and X axes are 9 and 125 μM, respectively). Coatings were stored at room temperature
589 in the dark at < 5% humidity under argon for 56 or 84 days (A, B) or air for 56 or 84 days (C, D).

590

591

592 **Table 1.** Hydrogen production by *R. palustris* coatings after storage for up to 56 days at 22 °C

593 and 60% humidity.

594

Storage period (days)	H_2 accumulation (% headspace)[a]		
	Sucrose[b]	Trehalose[b]	Sorbitol[b]
< 1	29.0 ± 0.5[d]	30.4 ± 6.4	31.9 ± 8.9
14	25.2 ± 1.9	21.3 ± 0.4	32.2 ± 12.6
28	3.3 ± 5.6	16.8 ± 14.5	0 ± 0
28[c]	19.3 ± 1.7	17.9 ± 0.4	0 ± 0
56	0 ± 0	0 ± 0	0 ± 0

595

596 [a]H_2 accumulation was measured 20 days after coatings had been rehydrated in PM(NF) medium

597 in closed vessels containing an argon atmosphere.

598 [b]Coatings contained glycerol plus the indicated osmolyte stabilizers

599 [c]Coatings were stored under <5% relative humidity; all others were stored at 60% humidity

600 [d]Values are averages from 3 replicate strips ± SD

601

602 **Table 2.** Hydrogen production by *R. palustris* coatings after storage for up to 56 days at 22 ºC

603 and <5% or 60% humidity.

604

Storage period (days)	H_2 accumulation (% headspace)[a]					
	Sucrose[b]			Trehalose[b]		
	<5%	60%	60%[c]	<5%	60%	60%[c]
7	51.1 ± 5.5[d]	9.4 ± 0.9	9.0 ± 2.4	35.2 ± 1.0	14.1 ± 3.4	9.3 ± 1.7
28	15.7 ± 0.7	0 ± 0	0 ± 0	20.2 ± 0.8	0.1 ± 0.1	0 ± 0
56	13.8 ± 4.4	0 ± 0	0 ± 0	18.5 ± 0.6	0 ± 0	0 ± 0

605

606 [a]H_2 accumulation in argon measured 20 days post rehydration in PM(NF)

607 [b]Coatings contained glycerol plus the indicated osmolyte stabilizer

608 [c]Coatings dried for 48 hours at <5% humidity, then stored at 60% humidity

609 [d]Values are averages from 3 replicate strips ± SD

610

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)	H_2 accumulation (% headspace)[a]			
	Sucrose (+glycerol)		Trehalose (+glycerol)	
	Air	Argon	Air	Argon
8	12.7 ± 0.3[c]	48.0 ± 7.8	9.8 ± 1.7	31.5 ± 4.2
8 (2nd flush)[b]	15.5 ± 2.3	36.3 ± 1.8	15.8 ± 2.0	30.3 ± 1.9
12	9.5 ± 1.0	69.1 ± 8.1	3.1 ± 1.0	52.3 ± 4.0

[a]H_2 accumulation in argon 10 days post rehydration in PM(NF) medium

[b]H_2 accumulation in argon (10 day incubation) following the initial H_2 production period (20 days) and one flushing/medium refresh event

[c]Values are averages from 3 replicate strips ± SD

628 **Supporting Information**
629

630 **Supporting Figure 1.** Illustration of the *R. palustris* latex coating method. (A) Cultures of *R.*
631 *palustris* grown anaerobically in serum vials; (B) Pellet of *R. palustris* cells from the 4 serum
632 vials; (C) Application of cell/latex/osmotic stabilizer mixture across the top of a pre-cut polyester
633 mask in a humidity-controlled chamber; (D) Spreading cell/latex mixture using a Mayer rod; (E)
634 Drying coatings in the pre-cut mask under air with humidity control; (F) Coating strips hydrated
635 in PM(NF) medium under argon headspace in Balch tubes
636

Fig. 1

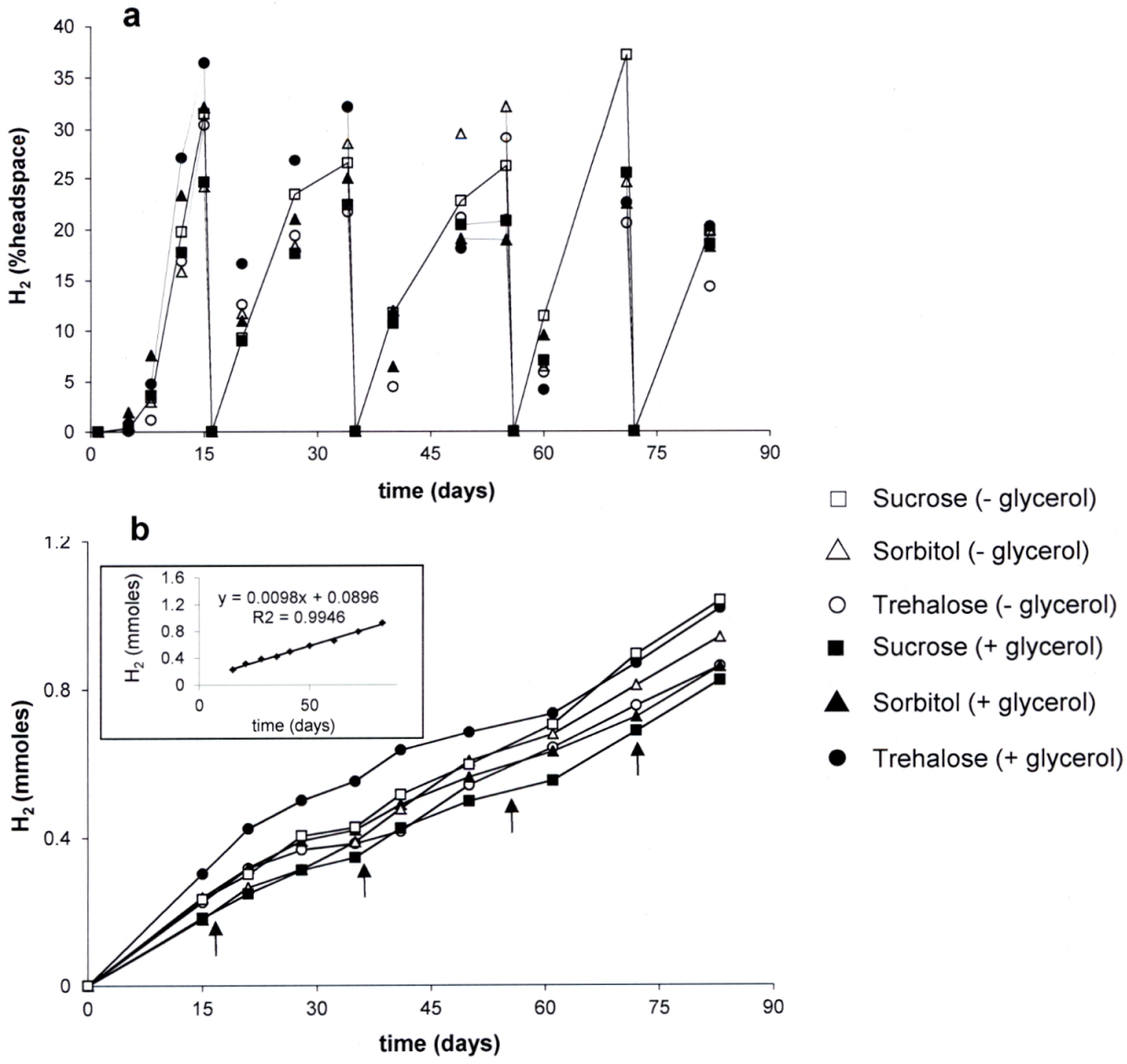
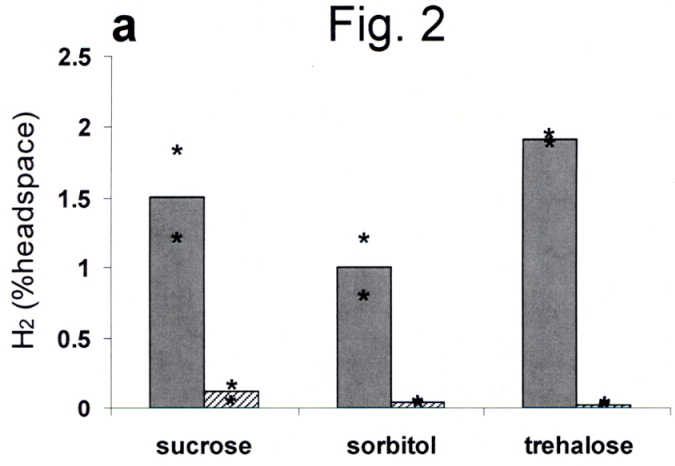
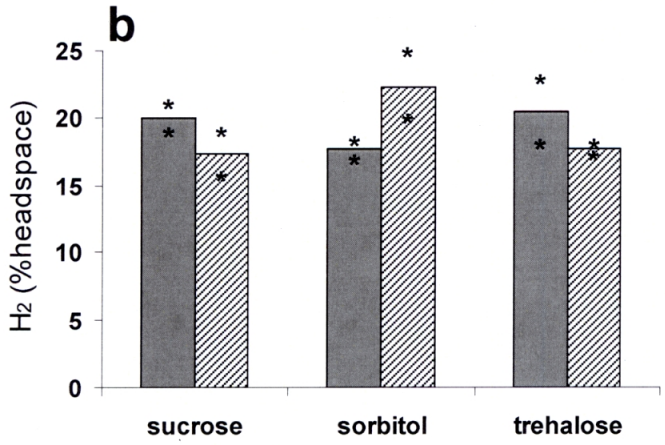

a
H_2 (%headspace)
time (days)
b
H_2 (mmoles)
time (days)
y = 0.0098x + 0.0896
R2 = 0.9946
Sucrose (- glycerol)
Sorbitol (- glycerol)
Trehalose (- glycerol)
Sucrose (+ glycerol)
Sorbitol (+ glycerol)
Trehalose (+ glycerol)

Fig. 2


a
2.5
2
1.5
1
0.5
0
H2 (%headspace)
sucrose
sorbitol
trehalose


b
25
20
15
10
5
0
H2 (%headspace)
sucrose
sorbitol
trehalose

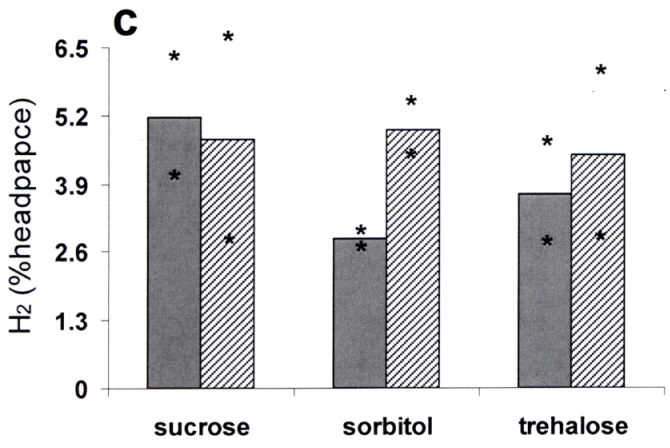

c
6.5
5.2
3.9
2.6
1.3
0
H2 (%headpapce)
sucrose
sorbitol
trehalose

Fig. 3

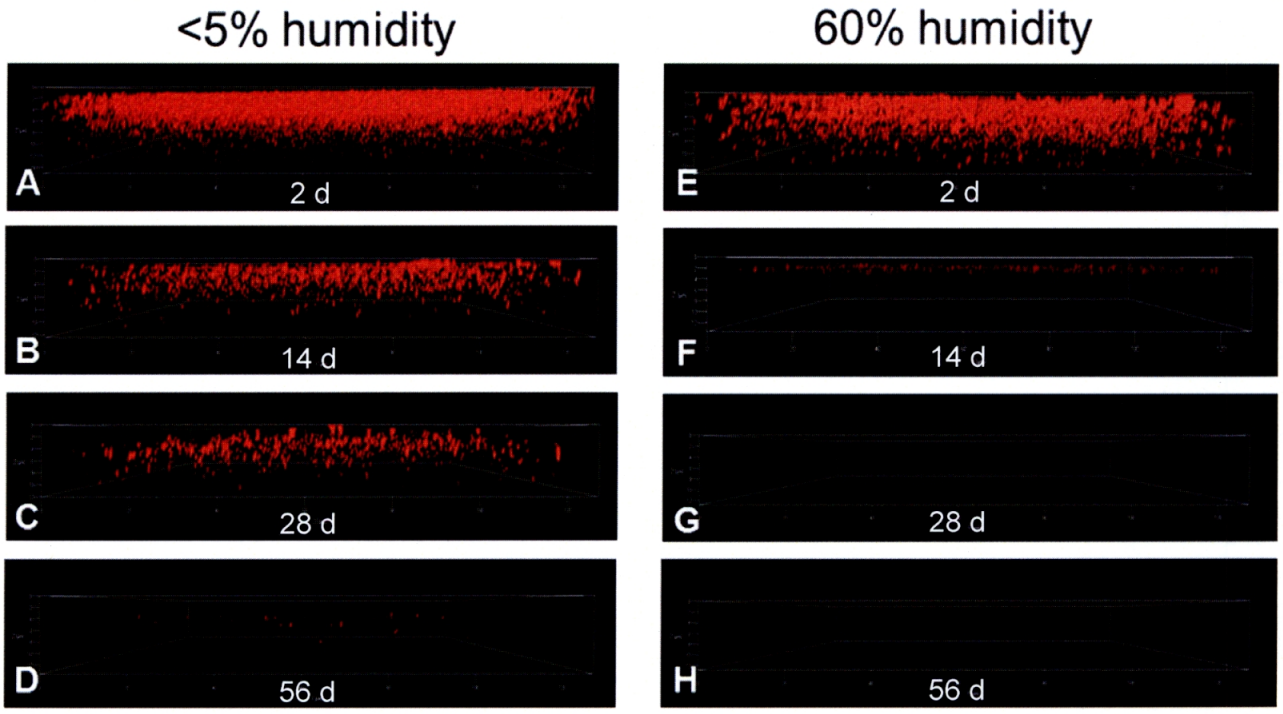
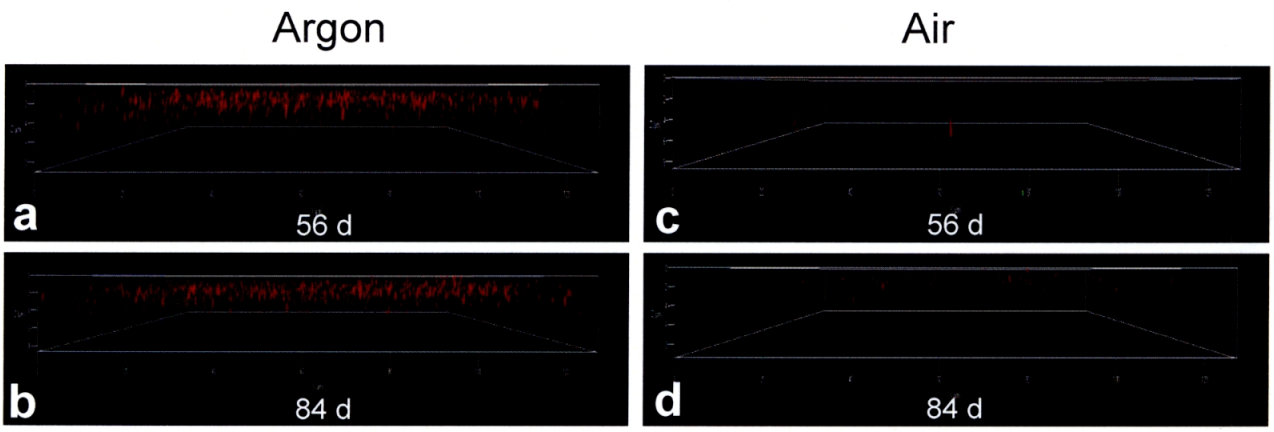


Fig. 4



Supp. Fig. 1