

Dark Fermentative Biohydrogen Production from Fructose-rich Resources by the Photosynthetic Purple Bacterium *Rhodospirillum rubrum*

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Dark Fermentative Biohydrogen Production from Fructose-rich

Resources by the Photosynthetic Purple Bacterium

Rhodospirillum rubrum

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Abstract

Background Photosynthetic purple bacteria are well known for biohydrogen production under phototrophic conditions, but industrial implementation is limited by inefficient light supply. For the purple nonsulfur bacterium *Rhodospirillum rubrum*, however, a growth medium and cultivation process are available for high-level expression of photosynthesis-related genes under semiaerobic dark conditions, completely independent of light. The cultivation regime opens the door to large-scale applications without the need for technically complicated and inefficient illumination in photobioreactors. Therefore, it was reasonable to evaluate the potential application of the specific phenotype for biohydrogen production in bioreactor processes with *R. rubrum* under semiaerobic and anaerobic dark conditions.

Results In this study, dark fermentative hydrogen production from fructose was investigated in stirred-tank bioreactors under semiaerobic and anaerobic conditions using defined medium as well as the fructose-rich agro-industrial residues pressed grape pomace juice and hydrolyzed molasses. The results consistently showed that oxygen limitation induced the full photosynthetic phenotype in the dark together with mixed-acid fermentation and hydrogen evolution in all bioreactor cultivations. Anaerobic operation consistently outperformed semiaerobic cultivation with respect to hydrogen yield and productivity. Hydrolyzed molasses gave the highest performance, achieving a volumetric productivity of $1.12 \text{ mmol H}_2 \text{ L}^{-1} \text{ h}^{-1}$, and

a biomass-specific productivity of $0.8 \text{ mmol H}_2 \text{ g}^{-1} \text{ h}^{-1}$ whereas defined medium yielded $0.49 \text{ mmol H}_2 \text{ L}^{-1} \text{ h}^{-1}$ hydrogen in fed-batch cultivation.

Conclusion The results demonstrate the feasibility of biohydrogen production from renewable, fructose-containing feedstocks using *R. rubrum* under semiaerobic and anaerobic dark conditions. Furthermore, quantitative reference data and descriptive performance metrics are provided for comparison with biohydrogen produced via photofermentation or from CO in *R. rubrum* and other photosynthetic purple bacteria.

Keywords Biohydrogen, Dark fermentation, Photosynthetic bacteria, Rhodospirillum rubrum, Resource Recovery

Background

The increased interest in biohydrogen as a cost-effective and clean alternative to fossil fuels is bringing photosynthetic purple bacteria (PPB) back into the spotlight of biological hydrogen research (e.g., [1–5]). PPB are exceptional in their metabolic versatility, enabling them to grow as photoheterotrophs, photoautotrophs, chemoheterotrophs or chemoautotrophs, depending on the environmental conditions. Anaerobically, they utilize light energy together with organic or inorganic electron donors to drive anoxygenic photosynthesis via cyclic photophosphorylation. The protein-pigment photosystem, comprising the reaction center and associated light-harvesting complexes, is embedded within large arrays of intracytoplasmic photosynthetic membranes (PMs) that are induced at low oxygen tensions. Under aerobic conditions, oxygen represses PM biosynthesis, and the cells shift to aerobic respiration. In the absence of both light and oxygen, anaerobic respiration and dark fermentative modes of metabolism are also possible. The metabolic flexibility is also reflected in hydrogen evolution pathways and mechanisms involving both light-dependent photofermentation and dark fermentation routes to H_2 [6].

However, the apparent advantage of using light as a resource comes with the major technical obstacle to efficiently supply light energy to the culture. At high cell densities and large cultivation volumes, light energy is always a limiting factor, preventing large-scale industrial application. In general, phototrophic growth rates are low, and high cell densities are not attainable in phototrophic processes.

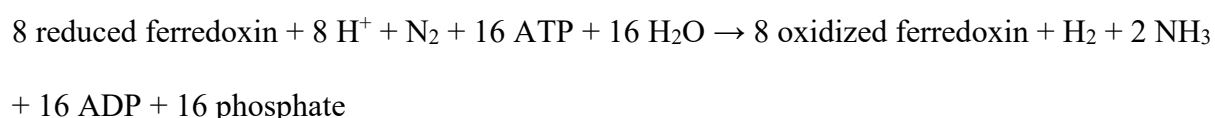
Interestingly, for the photosynthetic purple nonsulfur bacterium *Rhodospirillum rubrum*, a cultivation medium (M2SF medium) was designed that allows the full expression of PM and photosynthetic products in the complete absence of light under semiaerobic dark conditions [7, 8]. M2SF medium contains succinate and fructose as two carbon sources, where succinate is metabolized only by aerobic respiration, whereas fructose can be utilized both aerobically and by anaerobic fermentation [8]. The M2SF cultivation process allows the induction of the full photosynthetic phenotype in respiratory cells with high growth rates, high cell densities and large cultivation volumes [9] that are not attainable by anaerobic phototrophic or fermentative dark cultures.

The ability of *R. rubrum* to develop its photosynthetic apparatus in respiratory cells in the absence of light is closely associated with the response of the organism to oxygen availability and the cellular redox state. Experimental evidence [10] and simulation results of computational modelling [11] suggest a key role for components of the respiratory electron transfer chain as regulatory signals to induce the expression of photosynthesis-related genes under limiting oxygen conditions. In particular, the redox state of the cellular quinone pool (ubiquinone-10, rhodoquinone-10) pool responds to semiaerobic cultivation in the two-substrate M2SF medium in a way equivalent to light-signaling at low light conditions, where the induction of the photosynthetic apparatus is maximal. During semiaerobic metabolism in M2SF medium, electrons from aerobic respiration of succinate enter the quinone pool via the succinate dehydrogenase complex, with a further reduction simultaneously taking place via the reductive TCA cycle by anaerobic fermentation of glucose [8, 10]. The redox state of ubiquinones has

75 been previously identified to provide regulatory signals for controlling anaerobic gene
76 expression in *E. coli* [12] and the PPB *Rhodobacter capsulatus* [13]. The control of the
77 metabolic redox state imposed during semiaerobic cultivation in M2SF medium is therefore
78 critical for obtaining the desired phenotype. During semiaerobic growth in M2SF medium,
79 redox equivalents are generated by respiratory and fermentative metabolism, thereby creating
80 a unique physiological state in which aerobic respiration, photosynthetic membrane
81 development and dark fermentation coexist.

82 For *R. rubrum*, the first report of phototrophic evolution of molecular hydrogen dates back to
83 the middle of the last century [14]. The bacterium exhibits several pathways for biological
84 hydrogen production, including photofermentation and dark fermentation of organic electron
85 sources. A CO-dependent mechanism is also present in which H₂ formation by a water gas-shift
86 reaction is catalyzed by carbon monoxide dehydrogenase and hydrogenase [15, 16]. The CO-
87 dependent pathway is only induced in the presence of CO.

88 During photofermentation, nitrogenase is the primary H₂-evolving enzyme that catalyzes the
89 following reaction:



94 Here, electrons derived from the cyclic photophosphorylation electron transport system are
95 transferred by reverse electron transport via ferredoxin to hydrogenase and combine with
96 protons to molecular hydrogen.

97 Hydrogen formation in *R. rubrum* by dark fermentation of fructose and pyruvate has been
98 established in several previous studies, notably those of Schön and Biedermann [17], Voelskov
99 and Schön [18, 19] and Schultz and Weaver [20]. Under anaerobic dark conditions, the
100 formate:hydrogen lyase (FHL) enzyme complex has been demonstrated to be the dominant

source of generated H_2 [21, 22]. In this enzyme complex, formate is oxidized to CO_2 by formate dehydrogenase, and the resulting electrons are channeled to a [NiFe] hydrogenase for the reduction of protons to molecular hydrogen. Under oxygen-limited conditions, FHL is induced together with pyruvate formate lyase (PFL) [23] to provide formate ($HCOOH$) as a product of fermentative metabolism, according to

PFL: $Pyruvate + CoA \rightarrow Formate + Acetyl-CoA$

FHL: $HCOOH \rightarrow H_2 + CO_2$

There was only a minor contribution of nitrogenase in dark fermentation when phototrophic precultures allowed the light-activation of the enzyme [19]. Similar results were reported for microaerobic cultivation of *Rhodobacter capsulatus* JP91, where nitrogenase activity was $10 \times$ higher when dark-grown semiaerobic (microaerobic) cultures were illuminated prior to the nitrogenase assay [24]. Recently, for the first time, semiaerobic generation of hydrogen from M2SF medium has been demonstrated in small-scale cultures of *R. rubrum* [25].

Although dark fermentative hydrogen production by *R. rubrum* has been recognized for several decades, previous studies have primarily been conducted in small-scale laboratory cultures. While these investigations demonstrated hydrogen evolution from fructose and pyruvate and revealed the dominant role of the PFL/FHL pathway, they generally did not provide the quantitative process metrics required to evaluate the organism as a biotechnological production platform. In particular, systematic data on hydrogen yields, volumetric and biomass-specific productivities, and process performance in controlled stirred-tank bioreactors remain scarce. Moreover, direct comparisons between semiaerobic dark fermentation and anaerobic dark fermentation are lacking and the applicability to renewable fructose-rich agro-industrial feedstocks has not previously been demonstrated.

Therefore, the objective of the present study was to establish a quantitative benchmark for dark fermentative biohydrogen production by *R. rubrum* in controlled bioreactor cultivations. Specifically, we compared semiaerobic and anaerobic process stages using defined media and extended the process to the fructose-rich residues pressed grape pomace juice and hydrolyzed molasses. By reporting hydrogen yields, volumetric and biomass-specific production rates, and total hydrogen production under process-relevant cultivation conditions, this work provides quantitative performance data for comparison with previously reported photofermentative and dark-fermentative hydrogen-production systems while demonstrating, to our knowledge, the first application of these renewable feedstocks for biohydrogen production by *R. rubrum*.

Materials and methods

Organism and growth conditions

All experiments were conducted with the wild-type strain *Rhodospirillum rubrum* S1 (ATCC 11170).

Phototrophic anaerobic cultures were used to inoculate the bioreactors after cultivation in 100 ml Hungate flasks on a magnetic stirrer (Komet-Variomag poly, Thermo Fisher Scientific, Waltham, MA, USA) for 5 days at a stirrer speed of 150 rpm and a temperature of 30 °C. Illumination was provided by a set of tungsten 60 W light bulbs arranged regularly around the magnetic stirrer plate.

Bioreactor cultivations

Bioreactor cultivations were carried out in automated stainless-steel stirred-tank bioreactors (Biostat Cplus; Sartorius, Göttingen, Germany) with total volumes of 10 L and 30 L, respectively, at a temperature of 30 °C.

For fed-batch cultivation, a peristaltic pump was calibrated prior to each experiment to ensure accurate dosing. The feeding rate was then adjusted via gravimetric monitoring of the mass

decrease of the feed solution. BioPAT® MFCS/win 3.0 software was used for process monitoring and control.

Microaerobic conditions in the bioreactor were adjusted either by coupling the pO₂ to the pH of the cultivation as previously described [8] or by using an optical dissolved oxygen probe (VisiFerm DO 120, Hamilton, Reno, NV, USA) and a setpoint of 2.0 % air saturation with classical PID control. For anaerobic processes, the reactor was continuously purged with a stream of nitrogen gas (Nitrogen 5.0, N₂ ≥ 99.999 Vol, MTI IndustrieGase AG, Neu-Ulm, Germany).

All bioreactor cultivations were exploratory and conducted as single-runs with no replicates because of the technical complexity and high costs of large-scale bioreactor cultivations. More details are given in the results section.

All bioreactor cultivations were inoculated with a phototrophically grown pre-culture to a starting optical density of 0.1 (A₆₆₀ nm, 1 cm pathlength cuvette).

Analytical procedures

Growth and expression of intracytoplasmic photosynthetic membranes were monitored by absorption measurements at 660 nm and 880 nm, respectively, in 4 mm path length cuvettes (PMMA semi-micro cuvettes, VWR, Radnor, Pennsylvania, USA) using a Genesys 10 S UV–VIS photometer (Thermo Fisher Scientific, Waltham, MA, USA). In the results section, A₆₆₀ data were calculated for 1 cm path length for comparison with published data.

With complex feedstock such as molasses, this direct measurement was not possible because of turbidity and suspended solids. Therefore, 1 ml of culture broth was centrifuged (14.800 rpm, 5 min; Heraeus Fresco 21; Thermo Fisher Scientific, Waltham, Massachusetts, USA), and the cell pellet was subsequently washed with 1 ml of saline and centrifuged again. For the measurements, the cell pellet was resuspended in 1 ml of saline and appropriately diluted.

The quotient A_{880}/A_{660} represents the specific cellular amount of PM. The concentration of cell dry weight (CDW) was calculated using a correlation factor of $0.37 \text{ gCDW} \cdot A_{660}^{-1}$ (1 cm) for microaerobic cells with full pigmentation [26].

Organic acids (acetate, formate, succinate, propionate) were analyzed via high-performance liquid chromatography (HPLC) (Agilent 1100 Series, Agilent, Santa Clara, CA, USA) with a 250·4.6 mm RezexTM ROA-Organic Acid H⁺ column (Phenomenex, Torrance, CA, USA) with a particle size of 8 μm at a column temperature of 25 °C. The mobile phase consisted of 2.5 mmol L⁻¹ H₂SO₄ at a flow rate of 175 $\mu\text{L} \cdot \text{min}^{-1}$. The samples were recorded with a diode array detector (DAD) at 210 nm. In addition, a refractive index detector was used for the detection of fructose and glucose via the same HPLC system and method described above.

Hydrogen was monitored in the exhaust gas of the bioreactor via an online process gas chromatograph (Agilent 990 Micro GC (Agilent Santa Clara, CA, USA)) with argon as the carrier gas and two chromatographic separation channels, A and B. A: CP Molsieve 5 A 10 m column and B: a 10 m PoraPLOT U column. The column temperature was 40 °C for both channels. The sampling time was 10 s, and the collected sample was injected at 40 °C for 100 ms and analyzed at a static pressure of 150 kPa with a thermal conductivity detector. The run time per sample was 120 s. The concentration of hydrogen was determined via the slope of the single-level calibration with a calibration gas containing 993 ppm H₂ and 2010 ppm CO₂ in synthetic air (Westfalen AG, Münster, Germany).

The total amount of dissolved CO₂ and HCO₃⁻ in the fermentation broth was determined as total inorganic carbon using a TOC-VWP analyzer (Shimadzu, Kyoto, Japan).

Data processing for hydrogen determination

Data acquisition of the composition of the bioreactor gas phase with the software Agilent OpenLab CDS (Version 3.6) gives concentration units parts per million (ppm). Multiplying the

exhaust gas flow rate by the hydrogen volume fraction yields the volumetric flow rate of H₂ (L min⁻¹): $Q_{H_2} = Q_{gas} \times Y_{H_2}$

with

Y_{H_2} hydrogen volume fraction = ppm/10⁶

Q_{H_2} volumetric flow rate of H₂ (L min⁻¹)

Q_{gas} exhaust gas flow rate (L min⁻¹)

Conversion to a molar flow rate using the ideal gas law then requires the molar volume V_m with R : ideal gas constant (8.314 J/(mol·K); T : absolute temperature (K), and P : absolute pressure (Pa). The molar gas volume was corrected for $T = 30\text{ °C}$ (303,15 K) according to:

$$V_m = \frac{RT}{P} = \frac{(8.314 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}) \times (303.15 \text{ K})}{101325 \text{ Pa}} \times \frac{1000 \text{ L}}{1 \text{ m}^3} = 24.86 \text{ L} \cdot \text{mol}^{-1}$$

(instead of approximately 22.4 L mol⁻¹ under standard conditions).

To obtain a more accurate estimation of the total accumulated H₂ during fermentation, we summed the average rate during each sampling time interval.

$$V_{H_2, acc.} = \sum_{t=m}^{n-1} \left(\frac{Q_{H_2 m} + Q_{H_2 m+1}}{2} \right) (t_{m+1} - t_m)$$

Processing of biogenic residues

Pressed grape pomace juice (GPJ) from white wine grapes was supplied by RLP AgroScience GmbH, Neustadt, Germany. Prior to use, the raw material was processed as follows: the pH of the untreated GPJ was adjusted to 6.80 with 1 M NaOH, and the sample was subsequently

centrifuged at $4.400 \times g$ for 30 min at 4 °C. The supernatant was decanted into a Schott bottle and additionally filtered by cross-flow filtration (Sartorius AG SARTOFLOW® Slice 200 Benchtop Crossflow System) using a 0.2 µm Hydrosart filter membrane (0.1 m²). The filtrate was then again sterile-filtered via a 0.2 µm filter via a vacuum pump (Thermo Scientific Nalgene™ Rapid-Flow 75 mm Bottle Top Filter). The processed sterile GPJ was stored at 4 °C. Sugarcane molasses was purchased from EM-Chiemgau, Stephanskirchen, Germany. For microaerobic cultivation, a 10 % (w/v) solution of the original material was enzymatically hydrolyzed via incubation with 15 U/ml invertase from baker yeast (EC 3.2.1.26; Merck, Darmstadt, Germany) to release fructose from sucrose. The hydrolyzed and autoclaved material contained 15.97 ± 0.01 g L⁻¹ glucose and 22.41 ± 0.11 g L⁻¹ fructose, as determined by HPLC analysis (standard deviations from triplicate measurements) (for more details on the chemical composition of the sugarcane molasses, see, e.g., [27]). For cultivation, the fructose concentration was adjusted to 14.93 mmol L⁻¹ to replace the fructose from the original M2SF medium (16.7 mmol L⁻¹). For anaerobic fed-batch cultivation, the feeding mixture was composed of more concentrated hydrolyzed molasses with 96.70 mmol L⁻¹ glucose and 138.21 mmol L⁻¹ fructose, together with 400 mmol L⁻¹ sodium hydrogen carbonate.

Results

This study presents a series of bioreactor cultivations to estimate the capacity for fermentative biohydrogen production from fructose using the purple nonsulfur bacterium *Rhodospirillum rubrum*. The experiments were conducted under different levels of oxygen limitation under semiaerobic and anaerobic dark conditions.

Four different bioreactor cultivations are presented below. For the cultivations BCRruS1B and BCRruS1FB, fructose present in M2SF medium was used as carbon and electron substrate in a batch process (BCRruS1B) and a fed-batch process (BCRruS1FB), respectively (Fig. 1). With respect to more sustainable bioeconomic applications, two further cultivations were conducted

using biogenic residues from the food and agricultural industries as substrates. Biohydrogen production was tested with pressed grape pomace juice (GPJ) from white wine production (RLP AgroScience GmbH, Neustadt) (cultivation BCRruS1GPJ) and with molasses, a residue from sugar processing, after enzymatic hydrolysis by invertase (EC 3.2.1.26; cultivation BCRruS1MH).

Specifically, the cultivations were operated under the following conditions:

BCRruS1B: two-stage aerobic–anaerobic fermentation in M2SF medium without a microaerobic phase. After an aerobic growth phase, hydrogen evolution was induced in an anaerobic production phase. The pH was controlled with K_2CO_3 as a corrective agent to supply CO_2 for fructose breakdown (see below).

BCRruS1FB: M2SF medium with an initial aerobic growth phase followed by a semiaerobic growth and production phase and a subsequent anaerobic fructose/ $NaHCO_3$ fed-batch phase.

BCRruS1GPJ: pressed grape pomace juice (GPJ, diluted 1:10) as a carbon substrate with a semiaerobic growth and production phase, followed by pulse-like GPJ addition, anaerobic.

BCRruS1MH: enzymatically hydrolyzed molasses as a carbon substrate with a semiaerobic growth phase and feeding of hydrolyzed molasses/ $NaHCO_3$ in the subsequent anaerobic production fed-batch phase.

There were also some variations in culture conditions and process operation. The extent of oxygen limitation under semiaerobic conditions was not exactly identical among the four cultivations. A problem with the evaluation of microaerobic (semiaerobic) results is that this condition is not clearly defined but rather encompasses a window of different levels of oxygen limitation. However, in *R. rubrum*, the onset of semiaerobic growth is clearly indicated by the oxygen-sensitive induction of PM expression (increase in the A_{880}/A_{660} ratio). As the semiaerobic state approaches full anaerobiosis, the growth rates decrease to very low anaerobic levels. During semiaerobic growth, a mixed-mode metabolism with the induction of fermentative pathways along with simultaneous ATP generation by oxidative phosphorylation

results in much higher growth rates than achievable by full anaerobic fermentative metabolism [8]. Owing to the limitations of classical amperometric probes at very low dissolved oxygen (DO) levels, beyond approximately 5% air saturation, control of semiaerobic process operation was achieved either by applying the pH-DO strategy, introduced previously [8], or by PID control of pO_2 by a more sensitive optical DO probe (BCRruS1FB, BCRruS1MH). Despite the differences in DO control, the cultivation in M2SF medium provides two physiological indicators to clearly define semiaerobic conditions: (i) respiratory growth with specific growth rates between 0.04 and 0.07 h^{-1} (doubling time of 10–18 h), which is significantly greater than that attainable under fully anaerobic conditions, and (ii) the full induction of oxygen-repressed PM biosynthesis (determined as A_{880}/A_{660}) in growing respiratory cells. The sensor signal of the oxygen electrodes during the semiaerobic phase in all cultivations remained constant at pO_2 values between 0.0 and 2.0 % air saturation.

Fermentative biohydrogen production with defined media

For the cultivations BCRruS1B and BCRruS1F presented in Fig. 1, M2SF medium was used as the growth substrate for the aerobic and semiaerobic growth phases, respectively. In both fermentations, oxygen-limiting conditions induced the release of hydrogen, concomitant with the consumption of fructose.

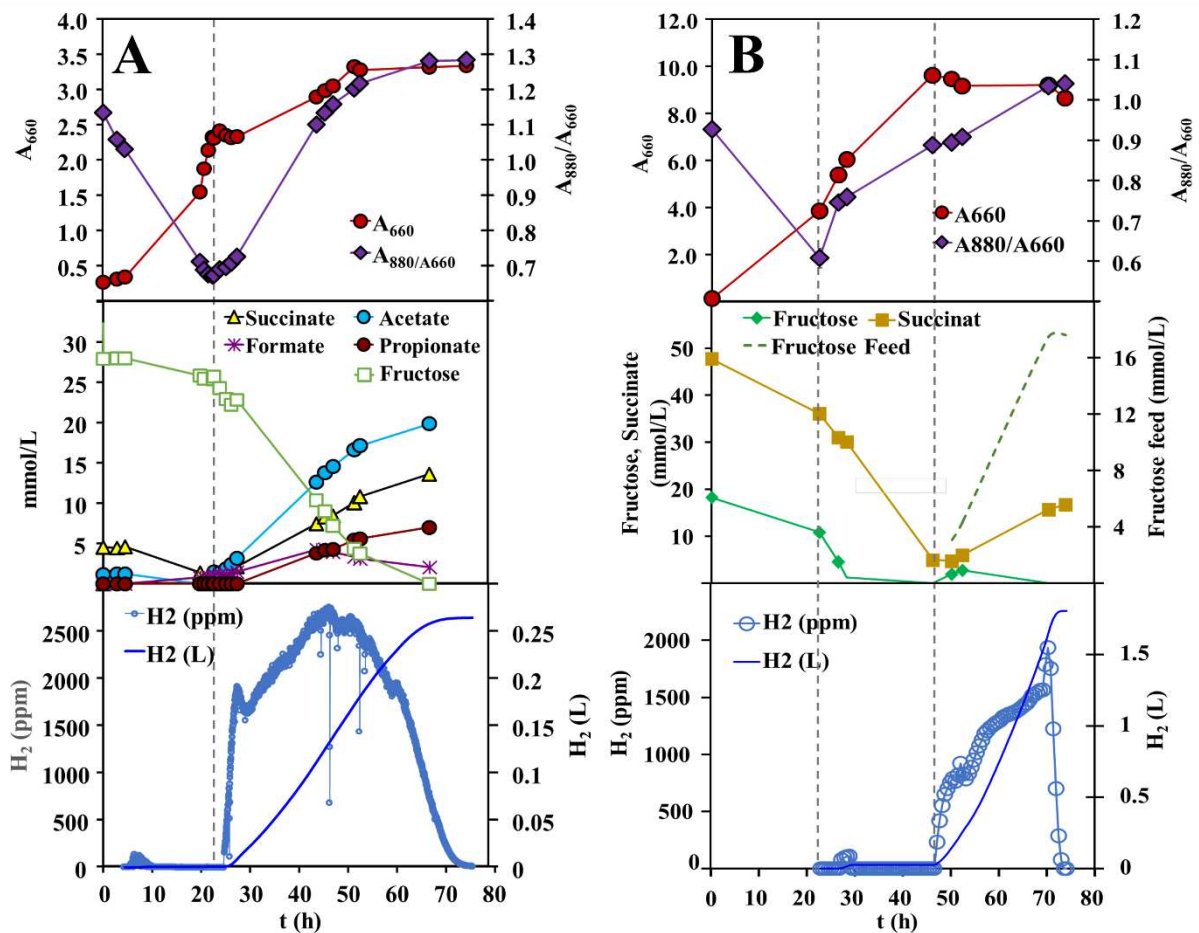


Fig. 1 Time courses of the bioreactor cultivation with defined media. A: Batch cultivation BCRruS1B; B: fed-batch bioreactor cultivation BCRruS1FB. Dashed lines indicate the onset of semiaerobic and anaerobic process stages, respectively. Fructose feed in (B) represents the total amount of fructose supplied during the anaerobic fed-batch stage. H_2 (L) represents the total amount of hydrogen accumulated throughout the cultivations.

In BCRruS1B, anaerobic conditions were applied after succinate of the M2SF medium was exhausted. During the anaerobic phase, the organic acids acetate, succinate, propionate and formate were excreted as fermentation products in addition to hydrogen, confirming previous reports on mixed-acid fermentation in *R. rubrum* [20, 28].

H_2 evolution was observed together with the induction of PM expression (A_{880}/A_{660}) after switching from aerobic to anaerobic (BCRruS1B) or semiaerobic (BCRruS1FB) conditions.

The maximum H_2 concentration in the exhaust gas occurred after 46 h of cultivation, when the fructose concentration fell below 10 mmol L⁻¹. From this point, a decrease in the hydrogen

production rate and excretion rate of organic acids was observed, which continued until fructose was depleted from the medium. The time courses illustrate that the observed hydrogen originated from fructose as a source of electrons and support the role of the PFL/FHL route as the primary source for fermentative hydrogen in *R. rubrum*.

However, *R. rubrum* possesses additional hydrogen-producing systems, such as nitrogenase and hydrogenases, including a Fe-only hydrogenase [29]. The extent to which alternative hydrogenases contributed to the observed hydrogen evolution is unclear. However, targeted gene expression analyses of the *nifH* gene, encoding the iron protein component of the nitrogenase enzyme complex expression by RT-PCR, showed transcription of *nifH* under semiaerobic dark conditions. Despite the discovery of a transcript, attempts to demonstrate enzyme activity by the acetylene reduction test remained unsuccessful. However, when semiaerobically grown cultures were transferred from dark to light conditions after growth had ceased, acetylene reduction was detected after 1 h of incubation with light exposure (supplemental Fig. S1).

Moreover, when semiaerobic cultures were grown with light in illuminated 2 L glass bioreactors, *nifH* transcription increased 1.78-fold compared to semiaerobic dark cultivation (supplemental Fig. S2). However, significantly more hydrogen was produced - specifically, 4.6 times the amount of the semiaerobic dark cultivation. The expression of formate hydrogen lyase (*fdhA*) in contrast was not increased by the application of light. These results are consistent with an earlier report that in *R. rubrum*, light-activation of nitrogenase is required for anaerobic dark cultures [19] and strongly suggest that the PFL/FHL route is a major source of hydrogen under the applied conditions.

It is further notable that in fermentation BCRruS1B, a redox electrode was applied to monitor the culture redox potential during the anaerobic process stage (Fig. 2A). In M2SF medium under anaerobic conditions, the excretion of molecular hydrogen decreased the culture redox potential to -550 mV compared with that of the standard hydrogen electrode (SHE). Although hydrogen

production was measured in the gas phase, the redox potential curve, recorded completely independently in the liquid phase, accurately matched even small fluctuations in the exhaust gas signal. Thus, under anaerobic conditions, a conventional redox SHE can be used to determine dissolved molecular hydrogen (partial pressure, pH_2), which may be useful for process control and optimization [30].

Fermentation BCRruS1FB had three separate stages: aerobic growth, semiaerobic growth and anaerobic production. After inoculation of 100 % air-saturated medium, the cultivation started with an aerobic phase without pO_2 control for consumption of dissolved oxygen and respiratory build-up of biomass. The onset of limiting oxygen supply at 42 h is indicated by the induction of PM expression (A_{880}/A_{660}), whereupon the semiaerobic pO_2 control was started to keep dissolved oxygen at 2 % air saturation. The specific growth rate during the semiaerobic phase was 0.039 h^{-1} , confirming the contribution of respiratory metabolism to biomass formation.

Although semiaerobic conditions were sufficient to induce hydrogen production, the subsequent anaerobic fructose-fed-batch phase resulted in a significant increase in the total amount of accumulated hydrogen (Fig. 1B). In addition to hydrogen, succinate was excreted as a fermentative product of commercial interest during the anaerobic fed-batch phase. In total, approximately 2 g of succinate and 1.81 L of hydrogen were produced during the fermentation. Interestingly, no hydrogen evolution could be detected in fructose fed-batch cultivations unless a certain amount of CO_2 was supplied simultaneously with fructose. CO_2 was added in the form of carbonate (K_2CO_3 as a pH corrective agent in BCRruS1B) or bicarbonate, ($NaHCO_3$) during the fed-batch phase in BCRruS1FB (concentration in the feed solution was 400 mM $NaHCO_3$, supplied with a feed rate of 17 mL h^{-1} (6.8 mmol h^{-1})).

The requirement for CO_2 to induce chemoheterotrophic hydrogen formation initially appears contradictory since the reduction of CO_2 competes for reducing equivalents, which are then lost for hydrogen production. However, these results are consistent with earlier findings that the degradation of fructose in *R. rubrum* is dependent on CO_2 [28]. For process control in a fed-

batch process it raises the question of which CO₂ supply optimally supports biohydrogen production and at what CO₂ content a detrimental effect occurs. To determine the optimal CO₂ supply, another fed-batch fermentation was conducted in which different HCO₃⁻ (bicarbonate) feed rates were sequentially tested for their effects on the hydrogen production rate. The highest productivity was observed when bicarbonate was fed at approximately 10 mmol h⁻¹. Both lower and higher feed rates had a negative effect on the production rate (Fig. 2B). Each applied HCO₃⁻ feed rate established a constant concentration of dissolved total inorganic carbon (CO₂ (aq) and HCO₃⁻) as well as a constant H₂ production rate. For example, the 10 mmol h⁻¹ feed rate corresponded to 1.6 mmol L⁻¹ total inorganic carbon. The experimental findings suggest a metabolic scenario for dark fermentative biohydrogen evolution in *R. rubrum*, as illustrated in Fig. 2C.

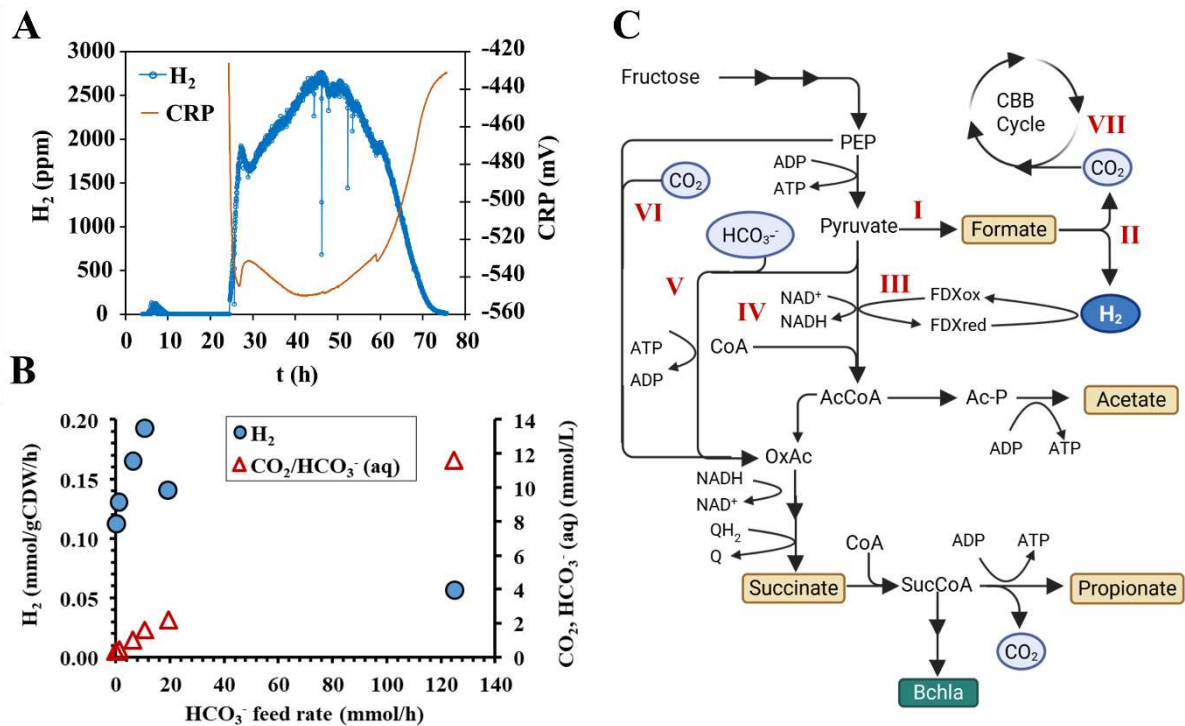


Fig. 2 A: Time course of hydrogen and culture redox potential during the anaerobic phase of BCRruS1B. **B:** Response of the hydrogen production rate (mmol/g cell dry weight/h) and concentration of total inorganic carbon (CO₂, HCO₃⁻) to varying feed rates of NaHCO₃ (CO₂). **C:** Simplified metabolic scenario with relevant reactions for dark fermentative hydrogen production from fructose in *R. rubrum*. Metabolites excreted during mixed acid fermentation are highlighted in brown

boxes. Relevant enzymes are numbered I: pyruvate formate lyase, EC:2.3.1.54, gene identifier Rru_A3000. II: Formate hydrogen lyase, EC 1.17.98.4, EC 1.17.98.5, Rru_A0324 (formate dehydrogenase); III: pyruvate-ferredoxin oxidoreductase (PFOR), EC:1.2.7.1, Rru_A2398; IV: pyruvate dehydrogenase complex, EC 1.2.1.104, Rru_A1880, Rru_A1879, Rru_A1215; V: pyruvate carboxylase, EC:6.4.1.1, Rru_A1121; VI: phosphoenolpyruvate carboxykinase (GTP), EC:4.1.1.32, Rru_A3419; VII: ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO), EC 4.1.1.39, Rru_A2400. Bchl_a represents bacteriochlorophyll *a* biosynthesis and photosynthetic membrane expression. PEP: phosphoenolpyruvate; FDX: ferredoxin (oxidized/reduced), AcCoA: acetyl-coenzyme A; Ac-P: acetylphosphate; OxAc: oxaloacetate; Q/QH₂: oxidized/reduced coenzyme Q10 (ubiquinone 10, rhodoquinone10); SucCoA: succinyl-coenzyme A; CBB cycle: calvin-benson-bassham cycle.

Fructose breakdown via the Embden–Meyerhoff pathway leads to pyruvate as a metabolic branch point of mixed acid dark fermentation. Previous studies have established that the PFL/HL route plays a major role in hydrogen evolution under this condition [21, 22]. Two enzymatic reactions are possible for the decarboxylation of pyruvate to acetyl-CoA. While the pyruvate dehydrogenase complex uses NAD⁺ in a physiologically irreversible reaction, the enzyme pyruvate:ferredoxin oxidoreductase (PFOR) catalyzes the reversible decarboxylation/carboxylation of acetyl-CoA using a low-potential ferredoxin as a redox cofactor. At present, it is unclear whether, under the applied fermentation conditions, PFOR contributes to the observed H₂ via electron transfer from ferredoxin to hydrogenase [22].

Under anaerobic conditions, some of the released CO₂ must be refixed to fuel the reductive branch of the tricarboxylic acid cycle, thereby leading to the formation of succinate and propionate. The excretion of propionate and acetate from their respective CoA-thioesters further allows the formation of additional ATP via substrate-level phosphorylation.

The anaplerotic carboxylations catalyzed by pyruvate carboxylase or phosphoenolpyruvate carboxykinase provide a straightforward explanation for the requirement to supply CO₂ (bicarbonate in BCRruS1FB, BCRruS1GPJ, BCRruS1MH, carbonate in BCRruS1B) to the bioreactor cultivations, since the metabolically released CO₂ was continuously stripped out of

the liquid phase by gas sparging of the reactor. As in the case of PFOR (reaction III in Fig. 2C), the contribution of ribulose 1,5 biphosphate carboxylase (Rubisco, reaction VII in Fig. 2C) to CO₂ refixation under the applied conditions is so far hypothetical and remains to be experimentally clarified. Note that the results shown in Fig. 2B depend heavily on the applied N₂-gassing rate, as high rates lead to the outgassing of dissolved CO₂(aq)/HCO₃⁻. At low gassing rates, however, the concentration of metabolically released CO₂(aq)/HCO₃⁻ remains sufficient to drive the refixation of CO₂. For the experiment shown in Fig. 2B, a lower gassing rate of 0.5 L min⁻¹ allowed biohydrogen formation even with a zero feed rate of NaHCO₃, whereas with the higher gassing rate of 1 mL min⁻¹ applied during BCRruS1FB, the additional supply of NaHCO₃ was essential.

Biohydrogen production from biogenic residues

The results of replacing or supplementing the defined M2SF medium with the complex biogenic residues pressed grape pomace juice (GPJ, BCRruS1GPJ) and hydrolyzed molasses (BCRruS1MH), respectively, are shown in Fig. 3.

Analysis of the chemical composition of GPJ suggested that it is a convenient feedstock primarily because of its high fructose content (approximately 500 mmol L⁻¹; equivalent to > 80 g L⁻¹, see supplemental Fig. S2). Furthermore, approximately 38 mmol L⁻¹ succinate was detected. The succinate concentration thus roughly corresponds to that of M2SF medium (40 mmol L⁻¹). With the detected amounts of succinate and fructose, both components needed in the defined M2SF medium to provoke the semiaerobic dark high-level expression of PM are present in high amounts in this residue. Preliminary shake-flask experiments using GPJ as a growth substrate revealed that no bacterial growth occurred in the original untreated material. The chemical composition (supplemental Fig. S3) suggests that the high osmolarity and high sugar concentrations are likely causes of the observed growth inhibition.

To avoid these inhibitory effects, further experiments were conducted with diluted GPJ. The bacteria could grow when GPJ was diluted 1:10, achieving approximately half the cell yield (A_{660}) obtained with M2SF medium (supplemental Fig. S3).

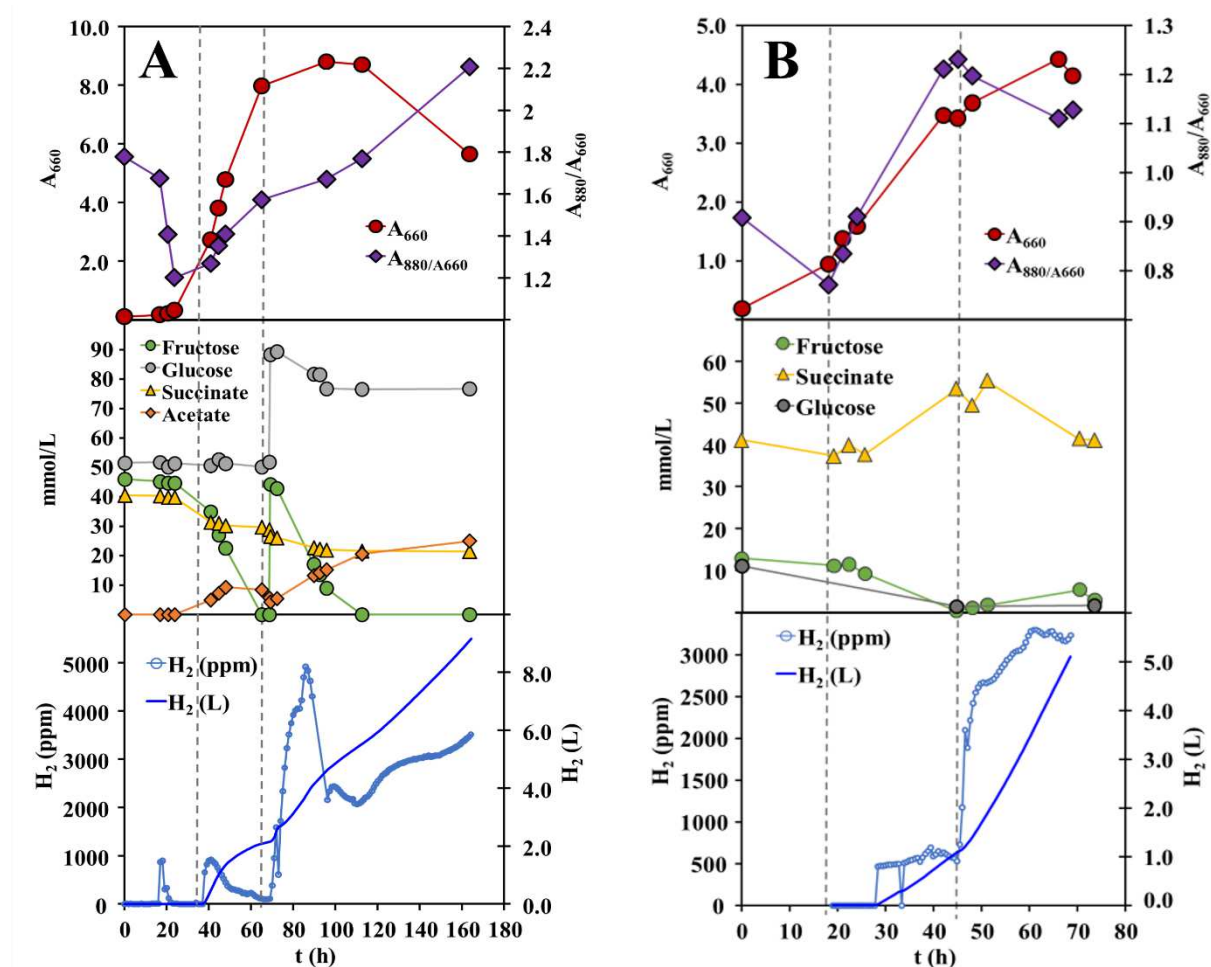


Fig. 3 Time courses of bioreactor cultivation with biogenic residues. A: BCRruS1GPJ; B: BCRruS1MH. The dashed lines indicate the onset of the semiaerobic and anaerobic process stages. H_2 (L) represents the total amount of hydrogen accumulated throughout the cultivation.

Both complex media completely reproduced the fully pigmented photosynthetic phenotype with high-level expression of PM (A_{880}/A_{660}) under semiaerobic dark growth conditions, which has thus far been observed only with the defined M2SF medium.

448 The cultivation process of BCRruS1GPJ using GPJ as a substrate is shown in Fig. 3A. Since no
449 growth occurred in undiluted GPJ, the feedstock was diluted 1:10 and supplemented with 40
450 mmol L⁻¹ ammonium succinate as a nitrogen source to account for the dilution of succinate.
451 The process again involves three phases: aerobic, semiaerobic and anaerobic. The anaerobic
452 phase started after the exhaustion of fructose. At this time point, a fed-batch pulse with
453 additional GPJ (incl. bicarbonate, see above) was applied, thereby increasing fructose to its
454 initial concentration and glucose to almost twice the initial value.

455 Similar to the M2SF medium, semiaerobic conditions led to the induction of PM expression
456 and biohydrogen production. The hydrogen concentration in the exhaust air followed the course
457 of the fructose concentration, thus again supporting the hypothesis that the hydrogen released
458 from GPJ originates from the fructose present in the residue. In total, 9.17 L of hydrogen was
459 produced during this fermentation.

460 For a further cultivation, BCRruS1MH, hydrolyzed molasses was used as a biogenic feedstock.
461 Molasses is produced in large quantities as a byproduct of the sugar processing industry. The
462 material typically contains approximately 50% sugar (primarily sucrose) along with vitamins,
463 minerals and nitrogen compounds [27]. Because of its nutrient profile and relatively low cost,
464 it is frequently selected as a feedstock for large-scale microbial fermentation. However, since
465 *R. rubrum* is unable to metabolize sucrose, it also cannot access the fructose contained in the
466 disaccharide. Therefore, molasses was enzymatically hydrolyzed to fructose + glucose prior to
467 fermentation by the addition of 15 U mL⁻¹ invertase (β -fructofuranosidase, EC 3.2.1.26) from
468 *Saccharomyces cerevisiae*. The hydrolysate was then used to replace fructose in M2SF medium
469 in the cultivation BCRruS1MH (Fig. 2B). The fructose content of the medium with hydrolyzed
470 molasses was 14.93 mmolL⁻¹, corresponding to the fructose concentration in the M2SF medium
471 (16.7 mmol L⁻¹). As expected from the invertase-catalyzed reaction, both hydrolysis products,
472 fructose and glucose were present at approximately the same concentrations in the hydrolysate
473 at the beginning of fermentation (Fig. 3B). After oxygen was consumed during the initial

aerobic batch phase, semiaerobic expression of PM was induced after approximately 20 h, as indicated by the increase in the A_{880}/A_{660} quotient. The specific growth rate in the microaerobic phase was $\mu = 0.054 \text{ h}^{-1}$. After 45 h, fructose was completely consumed, and the system was switched to anaerobic conditions. From this time point, a constant feed of hydrolyzed molasses (fructose, 138 mmol L^{-1} / NaHCO_3 , 200 mmol L^{-1} , 34 ml h^{-1}) was applied. Biohydrogen was released from hydrolyzed molasses during both the semiaerobic and the anaerobic phases. The production rates were 63 ml h^{-1} under semiaerobic conditions and 166 ml h^{-1} under anaerobic conditions.

Grape pomace juice and molasses were selected as agro-industrial residues because both contain high levels of fructose. Fructose is a component of M2SF medium and is readily metabolized by *R. rubrum*. However, it is known that in contrast to fructose, the wild-type strain *R. rubrum* S1 does not assimilate glucose under any condition [20, 31]. Both pomace juice and molasses contain large amounts of glucose in addition to fructose (Fig. 3 and supplemental Fig. S2). Interestingly, during the fermentations BCRruS1GPJ and BCRruS1MH, a significant decrease in glucose content was observed during the anaerobic phase (Fig. 3). After the pulse-like addition of GPJ to BCRruS1GPJ after 69 h, the glucose content increased immediately to ca. 90 mmol L^{-1} , after which it decreased to approximately 77 mmol L^{-1} (Fig. 3A). In BCRruS1MH, the measured glucose concentration decreased from $10.99 \text{ mmol L}^{-1}$ initially to 1.22 mmol L^{-1} at the time point when the semiaerobic growth phase ended and the anaerobic fed-batch phase started. During the anaerobic fed-batch phase, no accumulation of glucose was observed despite the continued addition of hydrolyzed molasses (Fig. 3B).

Although alternative explanations for the course of glucose cannot be ruled out, for example matrix effects with complex feedstock, the observed decrease in glucose is consistent with a recent report of glucose assimilation in the strain *R. rubrum* S1H [32]. In that study, glucose assimilation was observed under phototrophic conditions when glucose was supplied together with fructose but not when glucose was supplied as the sole carbon source. *R. rubrum* takes up

fructose through a PEP-dependent phosphotransferase mechanism [8, 31–33]. For glucose, an extracellular solute-binding protein (Rru_A0092) was significantly upregulated during the glucose assimilation phase and has been annotated as a potential glucose transporter [32]. However, its transport function has not been experimentally validated so far. In summary, the results of BCRruS1GPJ and BCRruS1MH, to our knowledge, represent the first demonstration of biohydrogen production from complex fructose-rich biogenic residues using *R. rubrum*.

Comparative assessment of key metrics

Several key metrics for comparative evaluation of the bioreactor cultivations conducted in the present study, are summarized in Table 1. The table is intended to allow an assessment of the chemoheterotrophic capacity for biohydrogen production in *R. rubrum* in comparison with published data on phototrophic and fermentative biohydrogen production in PPB.

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Table 1 Efficiency (yields) and productivity (rates) of the bioreactor cultivations. Substrates are sources of C6 carbon and electrons in the corresponding media. The yields Y_{H_2/C_6} for BCRruS1MH and BCRruS1GPJ were calculated from the total amount of hydrogen per unit of consumed hexose (fructose and glucose).

Fermentation	Substrate	Working vol. (L)	Condition	Y_{H_2/C_6}	H ₂ (mmol/L/h)	H ₂ (mL/L/h)	H ₂ (mmol/gCDW/h)	H ₂ (mL/gCDW/h)	H ₂ (L)
BCRruS1B	Fructose	4.00	anaerob	0.12	0.07	1.74	0.22	5.39	0.26
BCRruS1FB	Fructose	5.99 –	semiaerob	0.05	0.06	1.56	0.02	0.58	1.81
		6.23	anaerob	0.65	0.49	12.10	0.14	3.50	
BCRruS1MH	Molasses hydrolysate	5.95 –	semiaerob	0.33	0.43	10.71	0.50	12.42	5.09
		7.95	anaerob	1.03	1.12	27.77	0.80	19.94	
BCRruS1GPJ	Grape pomace juice	18.84 –	semiaerob	0.10	0.15	3.77	0.08	1.96	9.17
		24.16	anaerob	0.27	0.14	3.42	0.04	1.00	

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The following key observations can be drawn from the data presented in Table 1. With respect to fermentation conditions (semiaerobic vs. anaerobic), anaerobic operation generally resulted in higher hydrogen yields and production rates across all substrates than their semiaerobic counterparts. For example, in the fermentation BCRruS1FB, the anaerobic yield $Y_{H_2/\text{fructose}}$ reached 0.65, whereas under semiaerobic conditions, it was only 0.05.

Overall, molasses hydrolysate (BCRruS1MH) had the best performance under both semiaerobic as well as anaerobic conditions, achieving the highest hydrogen yield ($Y_{H_2/C6} = 1.03$), the highest volumetric productivity ($1.12 \text{ mmol H}_2 \text{ L}^{-1} \text{ h}^{-1}$) and the highest biomass-specific rate ($0.8 \text{ mmol H}_2 \text{ g}^{-1} \text{ h}^{-1}$).

The cultivation using grape pomace juice (BCRruS1GPJ) showed relatively low yields and productivity, although it generated the largest total hydrogen volume (9.17 L) because of the larger working reactor volume. Accordingly, the low specific productivity observed during the anaerobic phase results from the high biomass concentration attained at this stage (see A_{660} in Fig. 3A). At such elevated cell densities, hydrogen production does not appear to scale proportionally with the biomass concentration.

In summary, molasses hydrolysate delivered the highest specific and volumetric productivities, making it a promising substrate for industrial-scale hydrogen fermentation.

Discussion

The results presented here demonstrate the ability of the purple nonsulfur bacterium *R. rubrum* to produce biohydrogen from fructose-rich substrates in stirred-tank bioreactors under chemoheterotrophic dark conditions, thereby extending the published work on photoheterotrophic hydrogen production in *R. rubrum* and other PPBs. In contrast to classical dark fermentation by anaerobic bacteria, which requires the complete absence of oxygen, *R. rubrum* exhibits a unique semiaerobic physiological state in which fermentative metabolic

pathways and aerobic respiration operate simultaneously [8]. This metabolic configuration induces a fully developed photosynthetic phenotype in respiratory cells in the complete absence of light.

Compared with anaerobic fermentation, the semiaerobic state resulted in higher growth rates and supported substantial hydrogen formation even before full anaerobiosis. The complex substrates GPJ and MH fully induced the same PM phenotype as the defined M2SF medium.

Generally, fermentative hydrogen production yields lower product/substrate yields ($Y_{H_2/S}$) than phototrophic processes because of restrictions in anaerobic energy metabolism and redox balance. Photofermentation theoretically allows all electrons contained in the substrate to be directed to hydrogen, resulting in the highest theoretical yield with 12 H_2 /fructose. During photofermentation, light energy is converted into a proton motive force by cyclic photosynthetic electron transport, thereby enabling ATP synthesis and the generation of reduced electron carriers. Reduced ferredoxin can subsequently supply electrons to nitrogenase for proton reduction and H_2 evolution. Thus, the additional energy provided by photons for ATP generation and reductant formation is the fundamental energetic reason why photofermentation can achieve higher theoretical H_2 conversion. In contrast, in fermentative metabolism, substrate oxidation must support cellular growth, and ATP is generated predominantly by substrate-level phosphorylation. During fermentative metabolism, reduced or incompletely oxidized compounds (such as succinate and acetate) are inevitably excreted as byproducts in order to maintain redox balance (NAD^+ regeneration). Consequently, only a fraction of the reducing equivalents generated during substrate oxidation can be directed toward H_2 formation. In mixed-acid fermentation of *R. rubrum*, acetate, succinate, propionate, and formate were excreted (see BCRuS1B, Fig. 1A). In a scenario where all carbon would go to acetate and CO_2 , the theoretical yield decreases to 4 H_2 /fructose according to



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578 For hydrogen exclusively derived via the PFL/FHL route with two formates formed per
579 fructose, the maximum hydrogen yield is further reduced to only 2 H₂/fructose.

580 At present, it is unclear whether PFOR (reaction III in Fig. 2C) is present under the applied
581 conditions. The transfer of electrons from pyruvate via ferredoxin by PFOR and then to
582 hydrogenase would, however, not affect the maximal yield above. In real processes, for dark
583 fermentation of glucose or fructose to H₂ with anaerobic bacteria, yields of 1-2 can be achieved.
584 Each additional fermentation product, such as succinate or propionate, further reduces the
585 theoretical yield.

586 The energetic advantage of photofermentation is nevertheless accompanied by limited photon
587 availability in cultivation processes. Light penetration decreases strongly with increasing
588 biomass concentration and optical path length. The lower energetic yield of H₂ production in
589 dark fermentation is compensated by avoiding this limitation and permitting conventional high-
590 cell-density reactor configurations. From a technoeconomic perspective for low-value
591 applications such as biohydrogen, anaerobic dark fermentation of cost-effective abundant
592 biogenic residues is the most promising approach. The major process advantages are the lack
593 of light requirements, conventional reactor geometries, potentially high biomass
594 concentrations, and easier scale-up.

595 The results of BCRruS1GPJ and BCRruS1MH suggest that these complex agroindustrial
596 byproducts support robust hydrogen production under semiaerobic and anaerobic dark
597 conditions, respectively. On the basis of the key metrics given in Table 1, the BCRruS1MH
598 trial with hydrolyzed molasses was the most relevant setup. Molasses hydrolysate delivered the
599 highest specific and volumetric productivities, making it a promising substrate for industrial-
600 scale hydrogen fermentation. Both the yield as well as productivity of the cultivations with
601 defined media (BCrruS1B, BCRruS1FB) were significantly lower. Pressed grape pomace juice
602 (BCRruS1GPJ) also showed strong potential but lagged behind the productivity of hydrolyzed

603 molasses. Preprocessing and dilution were required for this feedstock. The analysis of the
604 chemical composition (supplemental Fig. S3) did not include, e.g., polyphenolic compounds.
605 Although white wines have a lower polyphenol content than red wines [34], it might be high
606 enough for the observed growth inhibition of undiluted GPJ. Nevertheless, biohydrogen
607 obtained from dark fermentation may have some potential for industrial valorization of
608 underutilized byproducts of wine grape processing [35].

609 The data of the present study were obtained from a series of bioreactor cultures with working
610 volumes of up to more than 20 liters (BCRruS1GPJ). Owing to the high technical complexity
611 and associated costs, these experiments were not performed in replicates and therefore lack
612 statistical analysis. Here, we merely wish to demonstrate that *R. rubrum* is capable of producing
613 biohydrogen from renewable resources via semiaerobic and anaerobic dark fermentation and
614 that the media with hydrolyzed molasses performed best in terms of yield and productivity.

615 A comparative evaluation of the present results with respect to academic literature data is
616 complicated by poorly defined conditions and calculations in many publications. Moreover,
617 different experimental setups and data processing make it difficult to compare results. For
618 example, in most published studies, the amount of accumulated hydrogen gas was determined
619 by gas chromatography in samples taken from the headspace of a closed cultivation vessel. In
620 contrast, our experimental setup applied constant gas purging of the bioreactor, thereby
621 allowing higher productivity by lowering the H₂ partial pressure. A complete raw data set for
622 BCRruS1MH of the present study is available in the supplemental material.

623 Some selected benchmark data from published reports for PPB biohydrogen are listed in Table
624 2 for comparison with the BCRruS1MH productivity. More comprehensive data are available
625 in a number of reviews on biohydrogen with PPB (e.g., Teke et al. 2023; Kapdan und Kargi
626 2006; Li et al. 2024; Faraloni et al. 2025).

627 **Table 2** Selected literature benchmarks for biohydrogen productivity by purple phototrophic bacteria compared with the wild-type *R. rubrum* S1 investigated in
628 this study.

Microorganism	Substrate / electron donor	Condition	Process mode	Volumetric productivity (mmol H ₂ L ⁻¹ h ⁻¹)	Specific productivity (mmol H ₂ g ⁻¹ h ⁻¹)	Reference
<i>R. rubrum</i> S1	Sugarcane molasses hydrolysate (BCRruS1MH)	Dark fermentation	Fed-batch	1.12	0.80	This work
<i>R. rubrum</i> UR2	Succinate	Photofermentation	Batch, continuous	0.84*	—	Melnicki et al., 2008
<i>R. rubrum</i> S1	Lactate (Glu as N source)	Photofermentation	Continuous	—	0.80*	Zürner & Bachofen, 1979
<i>R. rubrum</i> S1	CO	Dark fermentation	Fed-batch CO-feeding	27.2	—	Rodríguez et al., 2021
<i>R. palustris</i> P4	Glucose	Dark fermentation	Batch	—	29.9	Oh et al., 2002
<i>R. palustris</i> 42OL	Malate	Photofermentation	Semi-continuous	1.00*	—	Carlozzi, 2012
<i>R. palustris</i> DSM 127	Sugar beet molasses	Photofermentation	Batch	0.55	—	Sagir et al., 2017
<i>R. palustris</i> NCIMB 11774	Glycerol, Glu as N source	Photofermentation	Batch	—	0.59	Ross & Pott, 2021
<i>R. capsulatus</i> YO3	Sugar beet molasses	Photofermentation	Fed-batch	0.69	—	Oflaz & Koku, 2021
<i>R. sphaeroides</i> O.U. 001	Sugar refinery wastewater (sucrose + malate; Glu as N source)	Photofermentation	Batch	0.20*	—	Yetis et al., 2000
<i>R. sphaeroides</i> DSM 158	Lactate, Glu	Photofermentation	Batch	7.84*	—	Krujatz et al., 2015
<i>R. sphaeroides</i> YH01	Acetate	Photofermentation	Fed-batch	2.46*	—	Zhang et al., 2024

629 Note: Productivity values are the highest values reported in the respective studies. Volumetric productivity is standardized to mmol H₂ L⁻¹ h⁻¹ and biomass-
630 specific productivity to mmol H₂ g⁻¹ h⁻¹.
631 “*” Values originally reported as gas volumes were converted using V_m = 24.86 L mol⁻¹ (30 °C, 1 atm). “—” indicates that the respective metric was not

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634 Zürrer and Bachofen were the first to establish photosynthetic waste utilization with hydrogen
635 production in the wild-type strain *R. rubrum* S1 [36]. In batch cultures, the specific productivity
636 averaged $6 \text{ mL} \cdot \text{h}^{-1} \cdot \text{g}^{-1}$ dry cells using whey substrate. In continuous culture with lactate, it
637 reached $20 \text{ mL} \cdot \text{h}^{-1} \cdot \text{g}^{-1}$ which is the same range as the result of the dark fermentation
638 BCRuS1MH in the present study (Table 2).

639 A later synthesis–gas study demonstrated that *R. rubrum* S1 can produce hydrogen in darkness
640 from CO and H₂O by a water gas-shift reaction. The study also highlighted how the process can
641 be engineered with respect to agitation, CO partial pressure, batch versus fed-batch operation,
642 and dynamic gas supply. With CO-feeding, a peak production of $27.2 \text{ mmol CO-derived H}_2 \text{ L}^{-1}$
643 h^{-1} was reported [37]. However, CO-derived hydrogen production requires CO supply and
644 specialized reactor operation.

645 A *R. rubrum* mutant strain UR2, showing nitrogenase activity even in the presence of
646 ammonium [38] was used in the study of Melnicki et al. [39], with a maximum volumetric
647 productivity of $20.7 \text{ ml L}^{-1} \text{ h}^{-1}$.

648 A semicontinuous process for the reated PPB *Rhodopseudomonas palustris* 42OL resulted in
649 approximately $25 \text{ mL H}_2 \text{ L}^{-1} \text{ h}^{-1}$ (Carlozzi, 2012) in a 259 ml photobioreactor under intense
650 irradiation.

651 In another study, immobilized *R. palustris* NCIMB 11774 generated $14.7 \text{ mL g}^{-1} \text{ h}^{-1}$ in a
652 fluidized bed photobioreactor configuration. [40].

653 Yetis et al. demonstrated continuous hydrogen production from industrial wastewater by
654 *Rhodobacter sphaeroides* O.U.001 in a 0.4 l column photobioreactor [41]. When additional
655 carbon and nitrogen sources were added, the hydrogen production rate reached 0.005 L
656 $\text{hydrogen L}^{-1} \text{ h}^{-1}$. An engineered strain of *R. sphaeroides* with a hydrogen productivity of 61.2
657 $\text{mL L}^{-1} \text{ h}^{-1}$ from photofermentation of acetate under fed-batch conditions was reported by Zhang
658 2024 [42]. Sugar beet molasses was applied as photofermentative substrate for *Rhodobacter*

capsulatus YO3 by Oflaz & Koku in a 20 L tubular photobioreactor. The highest hydrogen productivity was 0.69 mmol H₂ L⁻¹ h⁻¹ obtained with molasses feeding (Oflaz & Koku, 2021). *R. capsulatus* YO3 is a strain in which the uptake hydrogenase gene was deleted [43]. Immobilized *R. capsulatus* YO3 gave 0.87 H₂ L⁻¹ h⁻¹ using a 1.4 L photobioreactor in a long-term sequential batch experiment (Sagir 2017). The highest productivity was reported by Krujatz et al. with *R. sphaeroides* DSM 158 grown on a mixture of glutamate and lactate in a one-liter photobioractor. Optimized process conditions with intense irradiation resulted in transient hydrogen production, with a peak of 195 mL L⁻¹ h⁻¹ hydrogen [44]. The examples cited demonstrate that the volumes involved are generally small and that extensive strain and process engineering contributed significantly to the results. Only few reports on biohydrogen and microaerobic (semiaerobic) conditions are available. Despite the scarcity of data, the potential for microaerobic biohydrogen production was acknowledged in a recently published review article [45]. For *R. rubrum*, the semiaerobic regime is particularly relevant because the organism can fully express its photosynthetic apparatus under oxygen-limited conditions even in darkness. Under semiaerobic dark conditions, respiratory metabolism can provide additional ATP through oxidative phosphorylation, while fermentative pathways simultaneously generate reducing equivalents and H₂. Respiration provides an energetic advantage for biomass formation; however, because a fraction of the substrate-derived electrons is consumed by respiratory metabolism rather than being directed toward H₂ formation, the H₂ yield is expected to remain below that achievable under an optimized anaerobic or photofermentative regime. Semiaerobic biohydrogen production with *R. rubrum* in M2SF medium has already been demonstrated using a new small-scale cultivation setup [25]. Another study investigated microaerobic (semiaerobic) dark fermentation in a strain of *Rb capsulatus* on the basis of the idea that a low amount of oxygen allows sufficient ATP production for the high energy

requirement of nitrogenase, thereby stimulating nitrogenase-mediated hydrogen production [24]. However, both reports do not include a comparison to anaerobic conditions and do not provide metrics for volumetric or specific production rates. Therefore, they are not included in Table 2.

The data in Table 2 show that the present study does not provide record-setting productivities; however, the results demonstrate biohydrogen production by dark fermentation in *R. rubrum*, even though no strain or process optimization has been carried out to date. Dark fermentation in *R. rubrum* is particularly attractive regarding the scalability to industrial levels - a feature that cannot be achieved with photoheterotrophic or CO-based processes.

In conclusion, although there is much space for the optimization of producer strains and process conditions, the valorization of carbohydrate (fructose)-containing agro-industrial sidestreams by semiaerobic and anaerobic dark fermentation with *R. rubrum* in a biorefinery concept may be an interesting option for circular bioeconomy applications. In addition to biohydrogen, a number of other photosynthetic products, such as PHA, coenzyme Q10, vitamin B12, 5-aminolevulinic acid and carotenoids [9] have become accessible by the semiaerobic and anaerobic cultivation regime applied here. Furthermore, PPB biomass itself is a valuable product that is rich in protein and vitamins for use as fertilizer or animal feed [46].

Conclusions

This study demonstrated that *R. rubrum* is an efficient producer of biohydrogen under chemoheterotrophic dark conditions in stirred-tank bioreactors, thereby providing an attractive alternative to light-dependent photofermentation.

Published studies on (photo)bioreactor cultivations typically do not include replicates, and corresponding statistical data are lacking. For example, when reviewing the literature to compare our bioreactor results, we found studies by Yetis et al. (2000), Krujatz et al. (2015), Sagir et al. (2017), Oflaz & Koku (2021), Rodriguez et al. (2021) (all listed in Table 2), and many others. All of these studies demonstrate that biological replicates (triplicates) are not standard practice for the complex and costly bioreactor experiments of this type. Even though the cultivations were performed as single runs, the overall results of the presented four different bioreactor cultivations presented allow some solid conclusions: Both semiaerobic and anaerobic process modes supported hydrogen evolution from fructose present in defined media or agro-industrial residues with anaerobic conditions generally yielding higher substrate-to-hydrogen conversion efficiencies and higher productivity. Semiaerobic conditions on the other hand enabled increased growth rates while maintaining substantial volumetric and specific productivities.

Importantly, the characteristic semiaerobic induction of the photosynthetic membrane system in the defined M2SF medium was fully reproduced with complex agro-industrial substrates. In terms of hydrogen production, hydrolyzed molasses outperformed the defined fructose media and delivered the highest yield and highest productivity, highlighting its strong potential for industrial application. Pressed grape pomace juice likewise supported robust hydrogen formation after suitable preprocessing, demonstrating the feasibility of valorizing fructose-rich residues from the food and wine industries.

Although fermentative yields remain inherently limited compared to photofermentation, the significantly higher growth rates, cell densities, simplified process control, and elimination of light requirements confer technoeconomic advantages. Overall, the presented quantitative metrics establish a benchmark for dark fermentative biohydrogen production with *R. rubrum* which awaits further optimization in the future.

Supplementary Information

Supplementary File

Author contributions

S. G. Conducted experiments, processing of the data, writing and editing.

J. B. Conducted experiments, processing of the data, writing and editing.

KC. B. Conducted experiments.

H.G. Responsible for project administration, funding acquisition, conceptualization of the study, conducted experiments, data processing, writing and editing.

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