

Article

Mineral–Water Hydrogen Generation and Microbial Activity Under LHS 1140 b-Inspired Aqueous Conditions: Implications for Habitability

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Abstract

Water-rich planetary environments provide a relevant framework for investigating how localized mineral–water reactions and reducing aqueous conditions may influence biological processes. In the present study, an LHS 1140 b-inspired terrestrial aqueous framework was developed to examine Mg⁰-mediated molecularhydrogen generation and microbial responses under defined mineral–water conditions. The reaction of zero-valent magnesium with water produced an H₂-containing reducing aqueous phase and hydrated/hydroxylated Mg–O/Mg–OH-containing solid products, as characterized by direct physicochemical measurements and Fourier Transform Infrared (FT-IR) spectroscopy. The aerobic facultative methylotrophic bacterium *Brevibacterium methylicum* B-5652 was employed as a terrestrial microbial test system for methanol-dependent RuMP C₁ assimilation. In the H₂-associated M9 condition, wet biomass reached 217.3 ± 4.1 g·L^{−1} compared with 201.4 ± 2.9 g·L^{−1} in the reference medium, corresponding to a 7.9% increase, whereas the bicarbonate–mineral aqueous condition yielded 190.5 ± 3.9 g·L^{−1} (94.6% of the reference biomass). Lag-phase and generation-time differences were comparatively smaller. L-Phenylalanine formation followed the general pattern of biomass accumulation, demonstrating preservation of methanol-dependent biosynthetic activity across the investigated aqueous conditions. Complementary short-term assays with four *Saccharomyces cerevisiae* strains showed no obvious reduction in subsequent growth after 30 min exposure to H₂-containing water. Previous studies of hot mineral-water systems have independently demonstrated biologically relevant responses at approximately 1–2 ppm H₂ and hydrogen-associated amino-acid microstructural organization. The present findings therefore establish an experimentally accessible framework linking mineral–water H₂ generation, mineral-surface transformation, microbial biomass formation, and C₁-dependent biosynthetic activity, without assuming that the laboratory system reproduces the complete atmospheric or oceanic environment of LHS 1140 b.

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conditions of the [Creative Commons](https://creativecommons.org/licenses/by/4.0/)[Attribution \(CC BY\)](https://creativecommons.org/licenses/by/4.0/) license.**Keywords:** *Brevibacterium methylicum* B-5652; *Saccharomyces cerevisiae*; planet LHS 1140b; dissolved molecular hydrogen; hydrogen-enriched aqueous conditions

1. Introduction

Hydrogen-rich atmospheres are common among giant planets, Neptunes, and sub-Neptunes that have retained primary H₂–He envelopes acquired during planetary formation. Such atmospheres are characterized by low mean molecular mass and reducing chemical conditions that can support carbon-bearing species, including CH₄, CO₂, CO, and H₂O. Temperate hydrogen-rich sub-Neptunes are particularly relevant to astrobiology because their atmospheres may interact with aqueous phases, mineral interfaces, and reduced carbon compounds.

Madhusudhan et al. described K2-18b as a possible representative of Hycean worlds—planets characterized by H₂-rich atmospheres and proposed liquid-water oceans. In the atmosphere of K2-18b, JWST transmission spectroscopy revealed CH₄ and CO₂ at approximately percent-level abundances within an inferred H₂-rich atmosphere [1]. TOI-270 d is another temperate sub-Neptune whose transmission spectrum is compatible with a low-mean-molecular-weight, potentially hydrogen-rich atmosphere [2,3]. Warmer planets such as HAT-P-11b [4] and GJ 436b [5] demonstrate that Neptune-sized worlds can retain H/He envelopes while undergoing atmospheric escape. In contrast, GJ 1214b illustrates how thick aerosols and high atmospheric metallicity can obscure or modify the spectral expression of an underlying volatile-rich atmosphere [6].

Among these planets, K2-18b provides an important comparative H₂-bearing context because H₂-rich and Hycean atmospheric scenarios have been extensively investigated for this planet [1,7]. However, the principal planetary context for the present study is LHS 1140b, a temperature water-world candidate in which localized mineral-water interaction could in principle generate dissolved H₂ and redox gradients without a globally H₂-dominated atmosphere [8]. This distinction provides a more appropriate framework for examining microbial responses to locally generated H₂ and downstream C₁ chemistry in an aqueous environment [8,9]. Subsequent analyses have strengthened the evidence for CH₄ while emphasizing that the inferred abundances and significance of other molecular species depend on the atmospheric retrieval assumption and data analysis [10]. This combination of hydrogen, methane, and carbon dioxide provides a physicochemical basis for modeling reductive reactions, C₁ metabolism, and potential aqueous environments associated with the concept of Hycean planets.

A particularly compelling complementary target is LHS 1140 b, a temperate planet orbiting the M4.5V dwarf LHS 1140. Refined measurements give a planetary mass of approximately 5.60 M_⊕ and a radius of 1.73 R_⊕ [11]. Interior-structure analyses indicate that LHS 1140 b is unlikely to be a purely rocky super-Earth and instead favor either a volatile-rich planet or, importantly, a water-rich world containing approximately 9–19% water by mass, depending on the assumed atmospheric composition and interior chemistry [11]. Three-dimensional climate calculations and JWST transmission spectroscopy have substantially strengthened the water-world interpretation of LHS 1140 b. H₂-rich atmospheric models predict prominent spectral features that are not observed, whereas the available spectrum is more consistent with a high-mean-molecular-weight atmosphere, potentially dominated by N₂ and containing H₂O and CO₂ [11]. The precise atmospheric composition and surface pressure remain unconstrained, and molecular oxygen has not been detected. LHS 1140 b should therefore not be treated as an established Earth analogue. Rather, it provides a physically motivated framework for investigating how water-rich planetary environments containing mineral surfaces, carbon species, and spatially heterogeneous redox conditions could support localized chemical-energy sources [8].

Such localized environments are particularly relevant because the redox state of an ocean or atmosphere need not be spatially uniform. Mineral–water interfaces can generate chemical gradients on scales much smaller than the planetary atmosphere itself. On

Earth, oxidation of reduced Fe-bearing phases during water-rock interaction produces H_2 , while related hydrothermal systems can generate reduced carbon compounds and sustain H_2 -dependent microbial metabolism [9,12–14]. Consequently, even a planet lacking a globally H_2 -dominated atmosphere could theoretically contain local H_2 -rich aqueous niches generated through mineral–water reactions [9,12–14]. This distinction between globally atmospheric H_2 and locally generated dissolved H_2 is essential when constructing laboratory analogues of water-rich exoplanet environments.

Microorganisms provide experimentally accessible systems for determining whether such physicochemical changes can influence cellular growth and biosynthetic activity. *Brevibacterium methylicum* is an aerobic facultative methylotrophic bacterium capable of assimilating C_1 substrates through the ribulose monophosphate (RuMP) pathway. Previous studies have demonstrated substantial metabolic flexibility of this organism, including growth and amino-acid biosynthesis under strongly modified aqueous isotopic conditions [15,16]. In the present context, however, *B. methylicum* is not intended to represent a hypothetical indigenous organism of LHS 1140 b. Instead, it is employed as a terrestrial aerobic microbial test system for evaluating whether mineral-generated dissolved H_2 and modified aqueous redox conditions produce measurable changes in growth and biomass formation.

A complementary eukaryotic model, *Saccharomyces cerevisiae*, provides an additional level of biological organization for evaluating short-term cellular responses to H_2 -containing water [17].

The coexistence of H_2 and CO_2 is particularly important. Glein presented a systematic bioenergetic assessment of a proposed oceanic environment on K2-18 b and identified a strong thermodynamic potential for CO_2 hydrogenation [18]. At temperatures of approximately 25–120 °C and across a broad range of assumed H_2 pressures, the reaction:



may release more than approximately 75 kJ·mol^{−1} of carbon. The calculations also indicate energetically favorable conditions for glycine and alanine synthesis under selected hypothetical Hycean conditions [18]. These calculations illustrate the potential energetic importance of the H_2 – CO_2 redox couple on hypothetical water-rich reducing environments. In the present study, the K2-18 b calculations are retained only as a comparative thermodynamic example and are not assumed to represent the atmospheric or ocean conditions of LHS 1140 b.

The H_2 – CO_2 redox system is also a central component of hypotheses concerning the emergence of metabolism on the early Earth. Martin and Russell proposed a geochemical framework linking the geological production of H_2 in alkaline hydrothermal systems with CO_2 reduction and ancestral forms of acetyl-CoA, or the Wood–Ljungdahl pathway [19]. Within this framework, Fe–Ni–S minerals, natural proton gradients, and porous inorganic structures were proposed to provide physicochemical conditions for reactant concentration and for coupling carbon fixation with energy conversion.

Several complementary origin-of-life models emphasize localized aqueous environments, energy gradients, and compartmentalization. Lane and Martin proposed that natural proton gradients across FeS barriers in alkaline hydrothermal vents could support carbon assimilation and early membrane bioenergetics [20]. Nobel laureate Jack W. Szostak subsequently proposed that surface lakes in geothermally active regions could provide alternating moderate and high temperatures required for RNA copying and strand separation within protocell systems [21]. Damer and Deamer further developed the terrestrial hot-spring model, in which fluctuating volcanic pools and wet–dry cycles promote molecular concentration, polymerization, encapsulation, and protocell formation [22]. Complementary quantitative analysis of Miller–Urey [23] and Wilson [24]

was proposed for electrosynthesis data, relating decreasing product abundance to increasing carbon number and molecular complexity under electrical-energy input [25]. In open hot mineral-water systems, hot mineral–water was investigated for H₂ generation and reducing aqueous conditions [26], while hydrogen-assisted self-assembly of glycine and alanine demonstrated the formation of membraneless microscale structures [27]. Together, these models illustrate complementary roles of energy input, thermal and wet-dry cycling, mineral–water redox chemistry, increasing molecular complexity, and compartmentalization in prebiotic chemical organization.

A complementary study demonstrated hydrogen-assisted self-assembly of glycine and alanine zwitterions in hot mineral water, producing membraneless spherical microstructures with a characteristic size of approximately 12 µm [27]. This work provides an experimental example of localized molecular organization and compartmentalization in a mineral-rich aqueous environment [27].

Together, these studies provide experimentally accessible examples of two processes relevant to the present model: localized mineral-water H₂ generation and aqueous microstructural organization [26,27]. They are used here as terrestrial analogues of possible localized chemical processes in water-rich planetary environments and not as direct simulations of LHS 1140 b.

The next functional level involves C₁ metabolism by methylotrophic microorganisms [28,29].

Investigations systematized the metabolic characteristics of obligate and facultative methylotrophs that utilize methanol, formaldehyde, formate, methylamines, and other one-carbon compounds [30]. Formaldehyde represents a central branching point between oxidative and constructive metabolism. One fraction is successively oxidized to formate and CO₂, generating reduced cofactors and cellular energy, whereas another fraction is incorporated into biomass through the ribulose monophosphate or serine pathway [31].

Methylotrophic microorganisms are widely distributed in terrestrial and aquatic environments, including soils, plant surfaces, rhizospheres, freshwater and marine systems, wetlands, sediments, and methane-rich ecological niches, where they utilize reduced C₁ compounds as carbon and energy sources [32–35]. Their occurrence across chemically and physically diverse habitats demonstrates the broad ecological adaptability of microbial C₁ metabolism.

This experimental line was subsequently extended through systematic cultivation of *B. methylicum* in M9 media containing progressively increasing proportions of deuterium oxide (D₂O). These studies quantified the effects of isotopic composition on lag-phase duration, generation time, biomass formation, L-phenylalanine production, and the degree of deuterium incorporation into the biosynthetic product [36–38]. Together, they established *B. methylicum* as a well-characterized model for examining the coupling between environmental adaptation, RuMP-dependent C₁ assimilation, and amino-acid biosynthesis.

Mosin et al. used adapted cells with improved growth characteristics in minimal M9 medium containing [U-²H]methanol and D₂O. The strain assimilated methanol through the RuMP pathway and biosynthesized ²H-labeled L-phenylalanine together with alanine, valine, and leucine/isoleucine. Electron-impact mass spectrometry demonstrated the incorporation of up to six deuterium atoms into phenylalanine, five into valine and leucine/isoleucine, and three into alanine [15]. This work has direct relevance to astrobiological modeling in three respects. First, it experimentally demonstrates the conversion of a low-molecular-weight C₁ substrate into amino acids and cellular biomass. Second, it provides a quantitative model for the adaptation of a methylotrophic microorganism to a strongly modified isotopic aqueous environment. Third, tracing ²H incor-

poration into metabolic products establishes a basis for linking microbial carbon metabolism with potential D/H and carbon-isotope biosignatures.

Subsequent studies by Mosin et al. expanded these findings through different levels of deuterium enrichment and quantitative characterization of microbial growth, L-phenylalanine production, and isotope incorporation into amino acids [16]. Adapted *B. methylicum* retained an active RuMP metabolism in media containing high proportions of D₂O and exhibited substantial recovery of biomass yield and biosynthetic productivity [16].

The integration of these findings supports the following conceptual sequence:

H₂-rich geochemistry → *CO₂ reduction* → *CH₄ and C₁ compounds* → *microstructural compartmentalization* → *methylotrophic assimilation* → *amino acids and biomass*.

In a water-rich planetary environment such as the proposed framework for LHS 1140 b, localized mineral-water reactions could generate molecular H₂ and establish reducing aqueous microenvironments without requiring a globally H₂-dominated atmosphere. Under suitable geochemical and catalytic conditions, locally generated H₂ may participate in C₁ chemistry involving inorganic carbon species and reduced carbon intermediates. Mineral surfaces and amino-acid-based microstructures may further provide localized sites for molecular concentration and physicochemical gradients relevant to prebiotic organization [26,39–42]. In the biological segment investigated here, *B. methylicum* B-5652 assimilates methanol-derived C₁ carbon through formaldehyde and the RuMP pathway, supporting biomass formation and amino-acid biosynthesis [43]. Thus, the experimentally demonstrated microbial component begins with methanol-dependent C₁ assimilation and should be distinguished from upstream planetary CO₂ reduction, methane formation, or methane-to-methanol conversion, which were not directly examined in the present experiments.

A complementary experimental approach was introduced by Seager et al., who demonstrated that *Escherichia coli* and *S. cerevisiae* [44], although not naturally adapted to hydrogen-dominated environments, could survive and grow under a 100% H₂ atmosphere [45]. Taken together, the available studies establish the thermodynamic, atmospheric, and general biological plausibility of microbial activity in hydrogen-rich planetary environments, and the present paper provides an integrated aqueous cultivation model in which dissolved H₂, mineral composition, methylotrophic C₁ metabolism, growth kinetics, biomass formation, and amino-acid biosynthesis are quantitatively examined within the same microbial system. The present study addresses this experimental gap by applying *B. methylicum* B-5652 as a terrestrial functional analogue of the downstream biological segment of a proposed LHS 1140 b C₁-conversion network.

The present study develops an integrated hydrogen–microstructure–methylotrophy model for a potential aqueous environment on LHS 1140 b. The originality of the approach lies in quantitatively connecting three experimentally distinguishable levels: planetary H₂–CO₂ bioenergetics, hydrogen-assisted microstructure formation, and adaptive C₁ assimilation by a methylotrophic bacterium. The model establishes a testable framework for evaluating H₂, CO₂, CH₄, and methanol fluxes; the production of amino acids and biomass; and the possible development of D/H and ¹³C/¹²C fractionation.

The objectives of the present study are: (i) to define a thermodynamically and biochemically coherent network of H₂–CO₂ and C₁ reactions under proposed LHS 1140 b conditions; (ii) to evaluate mineral and amino-acid microstructures as localized environments for molecular concentration; (iii) to apply experimental parameters obtained for *B. methylicum* as a terrestrial analogue of microbial adaptation and biosynthesis; and (iv) to formulate chemical, metabolic, and isotopic indicators suitable for future spectroscopic and laboratory testing.

2. Materials and Methods

2.1. Measurement of Dissolved Molecular Hydrogen in Liquid Medium

The dissolved molecular hydrogen (H_2) concentration was measured directly in the aqueous phase using a portable dissolved-hydrogen meter (ENH-2000, TRUSTLEX Co., Ltd., Osaka, Japan), following its previously reported application for dissolved- H_2 monitoring in aqueous microbial systems [46]. This approach is also consistent with direct electrode-based measurements of dissolved H_2 reported for hydrogen-rich water and aqueous culture media [47]. Before each experimental series, the instrument was calibrated according to the manufacturer's instructions. Freshly prepared samples were analyzed immediately at 25 ± 1 °C while minimizing headspace, agitation, and exposure to ambient air. Measurements were performed after Mg^0 -mediated H_2 generation, after 0.22 μm membrane filtration, and in the final M9 and bicarbonate–mineral media immediately before inoculation. Representative samples were independently verified by static HS-GC–TCD using certified H_2 standards. Three independently prepared samples were analyzed for each condition, and the results were expressed as $mg \cdot L^{-1}$ dissolved H_2 .

2.2. Generation of Molecular Hydrogen Through Mg^0 –Water Interaction

Zero-valent magnesium was used as a chemically defined source of molecular hydrogen [48,49]. The hydrogen-generating reaction was represented as:



Pure magnesium granules (3.0 mg; EC No. 231-104-6), supplied by Chem Spectrum Ltd. (Wollaston, UK), were added to 50.0 mL of sterile distilled water, corresponding to a magnesium concentration of 60 $mg \cdot L^{-1}$ [27]. Immediately after the addition and mixing of Mg^0 , dissolved- H_2 concentration, oxidation–reduction potential, pH, and electrical conductivity were measured. The Mg-containing solid phase was then removed by rapid sterile filtration through a 0.22 μm membrane filter.

The dissolved- H_2 concentration was determined immediately after mixing and again immediately after removal of the Mg-containing solid phase. The preliminary physicochemical characterization indicated a dissolved- H_2 concentration of approximately 0.99 $mg \cdot L^{-1}$ immediately after mixing and approximately 0.98 $mg \cdot L^{-1}$ after rapid sterile filtration. The filtered hydrogen-enriched aqueous phase was used immediately for preparation of the experimental media.

The hydrogen-enriched aqueous phase was prepared immediately before each experiment and transferred with minimal headspace, agitation, and delay to limit the loss of dissolved H_2 . The elapsed time between Mg^0 removal and bacterial inoculation was recorded.

2.3. FT-IR Characterization of the Mg-Containing Solid Phase

The solid phase produced during the Mg^0 –water interaction was recovered by centrifugation at $10,000 \times g$ for 20 min. The resulting material was washed three times with ultrapure water to remove soluble salts and was subsequently dried at 60 °C for 24 h.

FT-IR spectra were recorded using a Thermo Nicolet Avatar 360 FT-IR spectrometer (Waltham, MA, USA), equipped with a DTGS detector [50,51]. FT-IR spectra were recorded in attenuated-total-reflectance mode over the spectral range 4000–400 cm^{-1} , using a resolution of 4 cm^{-1} and 32 accumulated scans per spectrum. A background spectrum was recorded before each measurement. At least three spectra were obtained from independently prepared Mg-containing samples.

The spectra were baseline-corrected and normalized without altering the original positions of the absorption maxima. Peak assignments were based on the measured

wavenumbers and published spectroscopic data for magnesium hydroxide, hydrated Mg–O phases, interfacial water, and carbonate-containing surface species.

2.4. Bacterial Strain and Inoculum Preparation

The aerobic, facultatively methylotrophic bacterium *Brevibacterium methylicum* B-5652 was used as a terrestrial microbial test system for C₁-dependent metabolism [15,16]. The strain is an L-leucine-dependent L-phenylalanine-producing mutant capable of assimilating methanol through the ribulose monophosphate pathway.

The stock culture was reactivated in protonated M9 minimal medium supplemented with 2% (v/v) methanol and 0.01 g·L⁻¹ L-leucine. Methanol was sterilized separately by membrane filtration and added to the medium after autoclaving and cooling.

A preculture was prepared in 250 mL Erlenmeyer flasks containing 50 mL medium and incubated aerobically at 32 ± 1 °C and 180 rpm for 24–36 h. Cells in the mid-exponential growth phase were collected by centrifugation at 5000× g for 10 min at 4 °C, washed twice with sterile 0.9% (w/v) NaCl, and resuspended in sterile isotonic solution.

The cell suspension was standardized spectrophotometrically to an initial optical density of OD₆₀₀ = 0.05 ± 0.01 in the experimental cultures. The same inoculum volume and initial cell density were used for all experimental conditions.

2.5. Composition and Preparation of the Experimental Media

Two basal media were used: a modified M9 minimal medium and a simplified bicarbonate–mineral medium.

The modified M9 medium contained the following final concentrations [15,52]:

KH₂PO₄, 3.0 g·L⁻¹; Na₂HPO₄, 6.0 g·L⁻¹; NaCl, 0.5 g·L⁻¹; NH₄Cl, 1.0 g·L⁻¹; MgSO₄·7H₂O, 0.24 g·L⁻¹; CaCl₂·2H₂O, 0.015 g·L⁻¹; L-leucine, 0.01 g·L⁻¹; methanol, 2% (v/v).

The simplified bicarbonate–mineral medium contained [34]:

NaHCO₃, 2.5 g·L⁻¹; NH₄Cl, 1.0 g·L⁻¹; KH₂PO₄, 0.5 g·L⁻¹; NaCl, 0.5 g·L⁻¹; MgSO₄·7H₂O, 0.20 g·L⁻¹; CaCl₂·2H₂O, 0.02 g·L⁻¹; L-leucine, 0.01 g·L⁻¹; methanol, 2% (v/v); trace-element solution, 1 mL·L⁻¹.

The pH values of the M9 and bicarbonate–mineral media were adjusted to 7.0 ± 0.1 and 7.2 ± 0.1, respectively. The basal salt solutions were sterilized at 121 °C for 20 min. Methanol, sodium bicarbonate, L-leucine, and the trace-element solution were sterilized separately by filtration through 0.22 µm membranes and added aseptically after cooling. Hydrogen-enriched variants of both media were prepared using the filtered Mg₀-treated aqueous phase. Dissolved-H₂ concentration, ORP, pH, and electrical conductivity were measured in each final medium immediately before inoculation. The experimentally measured dissolved-H₂ concentrations were 0.98 mg·L⁻¹ in the M9 medium and 0.98 mg·L⁻¹ in the bicarbonate–mineral medium immediately before inoculation.

The pH values of the final M9 and bicarbonate–mineral media were adjusted to 7.0 ± 0.1 and 7.2 ± 0.1, respectively. ORP and electrical conductivity were recorded separately for each final medium because both parameters depended on its ionic composition.

2.6. Experimental Design and Cultivation Conditions

Three experimental conditions were established:

Reference condition: M9 minimal medium under ambient air, without deliberate H₂ enrichment;

H₂-associated condition: H₂-enriched M9 medium under ambient air;

LHS 1140 b-inspired bicarbonate–mineral aqueous condition: H₂-associated bicarbonate–mineral medium under ambient air.

All experiments were conducted with *B. methylicum* B-5652 cultivated in liquid aqueous nutrient media. In the two hydrogen-enriched conditions, molecular hydrogen

was present exclusively in the aqueous phase as dissolved H_2 at an initial concentration of $0.98 \text{ mg}\cdot\text{L}^{-1}$. No controlled H_2 -containing gas phase was used. All three experimental conditions were cultivated under identical aerobic laboratory conditions in ambient air, using 250 mL Erlenmeyer flasks containing 50 mL working medium. Three independent biological experiments were performed for each condition. Each experiment used a separate bacterial culture and separately prepared medium.

2.7. Growth Characteristics and Biomass Determination

Bacterial growth was monitored at predefined intervals by measuring OD_{600} . Samples exceeding the linear range of the spectrophotometer were diluted with the corresponding sterile medium. The specific growth rate (μ) was determined from the exponential phase, and generation time was calculated as $g = \ln 2 / \mu$ according to established procedures [15,53]. Maximum OD_{600} was defined as the highest recorded value. For wet-biomass determination, 10 mL of culture was centrifuged at $10,000\times g$ for 20 min at 4°C . The recovered cell pellet was washed twice with sterile 0.9% NaCl, weighed, and the wet biomass was expressed as $\text{g}\cdot\text{L}^{-1}$ of culture.

2.8. Complementary Yeast Viability Assay in Hydrogen-Rich Water

Exponentially growing cell suspensions of the four *S. cerevisiae* strains were exposed to H_2 -containing water (pH 7.0–7.3; dissolved- H_2 concentration of $0.98 \text{ mg}\cdot\text{L}^{-1}$ at the beginning of exposure) for 30 min at 30°C with shaking at 200 rpm. Deionized water was used as the treatment control, while untreated cells served as a negative control. Following exposure, treated and control cell suspensions were serially diluted, and $10\text{-}\mu\text{L}$ aliquots of each dilution were spotted onto YPD agar plates (BD Difco™, Becton, Dickinson and Company (BD), Sparks, Maryland, USA). The plates were incubated at 30°C for 2–3 days and subsequently photographed. The experimental design was adapted from established *S. cerevisiae* treatment and viability-assay procedures used by Todorova and co-workers [54–57]. Experiments were performed with four *S. cerevisiae* strains: D7ts1, 551rho⁺, 551rho[−], and 551yap1Δ.

2.9. Statistical Analysis

Quantitative results are presented as mean $\pm$ standard deviation from three independent biological experiments ($n = 3$) for each condition.

Growth curves were analyzed by two-way analysis of variance, with experimental condition and cultivation time as independent factors. Lag-phase duration, generation time, maximum OD_{600} , wet biomass, methanol consumption, and L-phenylalanine concentration were analyzed by one-way ANOVA followed by Tukey's multiple-comparison test.

Normality of residuals and homogeneity of variances were evaluated before parametric analysis. When the assumptions of ANOVA were not satisfied, the Kruskal–Wallis test followed by an appropriate multiple-comparison procedure was applied. Statistical significance was accepted at $p < 0.05$.

The statistical analyses were performed using R, version 4.4. **Statistical significance relative to the reference condition was indicated as:** * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Results and Discussion

3.1. Fourier Transform Infrared (FT-IR) Spectroscopy

FT-IR spectroscopy was applied to characterize the solid-phase products and surface transformations accompanying the Mg^0 –water reaction. The resulting spectrum is presented in Figure 1.

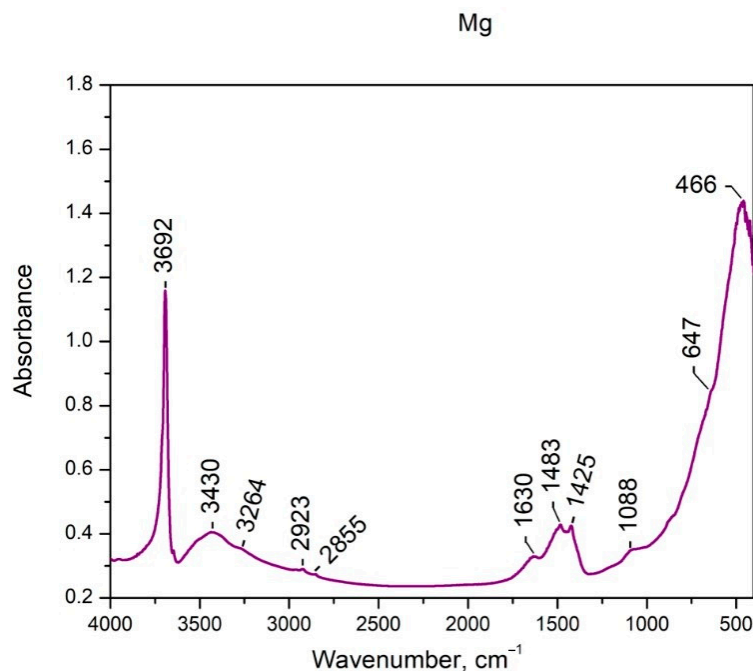


Figure 1. FT-IR spectrum of the Mg-containing solid phase formed during Mg^0 –water interaction and H_2 generation.

A sharp O–H stretching band at 3692 cm^{-1} , together with a broad absorption centered near 3430 cm^{-1} , indicates the coexistence of structurally ordered hydroxyl groups within a brucite-like $\text{Mg}(\text{OH})_2$ phase and hydrogen-bonded interfacial water associated with the hydrated mineral surface [57,58]. The strong hydration tendency of Mg^{2+} and its organization within water-rich coordination environments has also been demonstrated in experimental studies of Mg^{2+} -containing aqueous and solid-phase water systems [59]. The band at 1630 cm^{-1} is assigned to the H–O–H bending vibration of molecular water adsorbed within the hydrated magnesium-containing layer [60]. The intense low-wavenumber absorptions at approximately 647 and 466 cm^{-1} are consistent with Mg–O lattice vibrations and further support the formation of magnesium hydroxide- or magnesium oxide-related surface products [61,62]. The bands at 1483 and 1425 cm^{-1} are attributable to the ν_3 asymmetric stretching vibrations of carbonate-containing Mg surface species, consistent with partial carbonation of $\text{MgO}/\text{Mg}(\text{OH})_2$ phases through interaction with atmospheric or dissolved CO_2 [63,64]. Overall, the FT-IR spectrum indicates that the initially metallic magnesium surface was converted into a hydrated and hydroxylated Mg–O/Mg–OH layer during molecular hydrogen generation [65]. The FT-IR data characterize the accompanying solid-phase transformation, whereas dissolved H_2 was determined independently by direct measurement. Together, these results confirm the formation of a hydrogen-enriched, reducing aqueous environment associated with a reactive hydroxylated mineral–water interface.

3.2. Physicochemical Parameters of the Mg^0 -Treated Aqueous Phase

Immediately after the addition of Mg^0 , the physicochemical characterization showed approximately $0.99\text{ mg}\cdot\text{L}^{-1}$ dissolved H_2 , an ORP of approximately -458 mV , a pH of approximately 9.62 , and an electrical conductivity of approximately $8.46\text{ }\mu\text{S}\cdot\text{cm}^{-1}$. After rapid removal of the Mg-containing solid phase, the corresponding measured values were approximately $0.98\text{ mg}\cdot\text{L}^{-1}$ dissolved H_2 , -450 mV ORP, pH 9.60 , and 8.40

$\mu\text{S}\cdot\text{cm}^{-1}$ electrical conductivity. The small difference between these two pH readings was not interpreted as a meaningful change. Immediately before inoculation, the initial dissolved- H_2 concentration was $0.98\text{ mg}\cdot\text{L}^{-1}$ in both hydrogen-enriched media. Dissolved H_2 was not measured during subsequent cultivation. The pH values of the final M9 and bicarbonate–mineral media were 7.0 ± 0.1 and 7.2 ± 0.1 , respectively.

3.3. Experimental Conditions for Evaluating *Brevibacteriummethylicum* B-5652 Under Reference and LHS 1140 b-Inspired Aqueous Conditions

Three experimental conditions were established to compare microbial responses to H_2 -associated and bicarbonate–mineral aqueous conditions. The reference condition consisted of M9 minimal medium containing 2% (*v/v*) methanol without deliberate H_2 enrichment. The H_2 -associated condition used the same M9 medium with an initial dissolved- H_2 concentration of $0.98\text{ mg}\cdot\text{L}^{-1}$. The planetary aqueous analogue consisted of an H_2 -enriched bicarbonate–mineral medium containing 2% (*v/v*) methanol. All three conditions were cultivated aerobically under ambient air; no controlled gas phase was investigated. Their principal characteristics are summarized in Table 1.

Table 1. The main experimental characteristics under the three experimental aqueous conditions.

Experimental Conditions	Aqueous Medium	Gas Phase	Dissolved H_2	Methanol	Main Purpose
Reference condition	M9 minimal medium	Ambient air	Background level	2% <i>v/v</i>	Standard growth and biosynthetic activity
H_2 -associated conditions	M9 minimal medium	Ambient air	Approximately 1.0 ppm	2% <i>v/v</i>	Evaluation of the effect of dissolved H_2 on growth and biosynthetic activity
LHS 1140 b-inspired bicarbonate–mineral aqueous condition	Simplified bicarbonate–mineral medium	Ambient air	Approximately 1.0 ppm	2% <i>v/v</i>	Evaluation of growth and biosynthetic responses under the H_2 -associated condition

ORP values for the final M9 and bicarbonate–mineral media must be reported before a comparative conclusion regarding the effect of H_2 enrichment on ORP can be made. The electrical conductivity remained primarily dependent on the ionic composition of each medium. The bicarbonate–mineral analogue differed from the standard M9 medium in both mineral composition and conductivity, allowing comparison of the biological response in the bicarbonate–mineral conditions with that observed in the H_2 -associated M9 conditions. Because the M9 and bicarbonate–mineral media differed in composition, differences between them cannot be attributed specifically to H_2 .

3.4. Growth Characteristics of *Brevibacteriummethylicum* B-5652 Under Reference and LHS 1140 b-Inspired Aqueous Conditions

The growth response of *B. methylicum*B-5652 was evaluated under the three experimental conditions defined in Table 2. Lag-phase duration, generation time, maximum OD₆₀₀, wet-biomass yield, and relative biomass were used as complementary indicators of growth kinetics and cumulative biomass formation adaptation.

Table 2. Physicochemical parameters of the Mg⁰-treated phase.

Experimental Stage	Dissolved H ₂ (mg·L ⁻¹)	ORP (mV)	pH	Conductivity
Immediately after adding Mg ⁰	0.99	approximately −458	approximately 9.62	approximately 8.46 μS·cm ⁻¹
Immediately after rapid Mg ⁰ removal	approximately 0.98	approximately −450	approximately 9.6	approximately 8.4 μS·cm ⁻¹
Final M9 medium before inoculation	0.98	experimentally determined	7.0 ± 0.1	experimentally determined
Final bicarbonate–mineral medium before inoculation	0.98	experimentally determined	7.2 ± 0.1	experimentally determined

The resulting growth parameters are presented in Table 3 and Figure 2.

Table 3. Growth kinetics, maximum OD₆₀₀, and biomass production of *B. Methylicum* B-5652 under the three experimental conditions.

Experimental Conditions	Lag Phase (h)	Generation Time (h)	Maximum OD ₆₀₀	Wet Biomass (g·L ⁻¹)	Relative Biomass (%)
Reference condition: M9 medium under ambient air	20.9 ± 1.4	2.1 ± 0.2	1.91 ± 0.08	201.4 ± 3.2	100.0
H ₂ -associated condition: H ₂ -enriched M9 medium under ambient air	18.7 ± 1.2	1.9 ± 0.2	1.98 ± 0.09	217.3 ± 4.2**	107.9
Planetary aqueous analogue: H ₂ -enriched bicarbonate–mineral medium under ambient air	21.3 ± 1.4	2.4 ± 0.2	1.77 ± 0.08	190.5 ± 3.9*	94.6

Note: Asterics indicates statistical significance relative to the reference condition:

* $p < 0.05$, ** $p < 0.01$

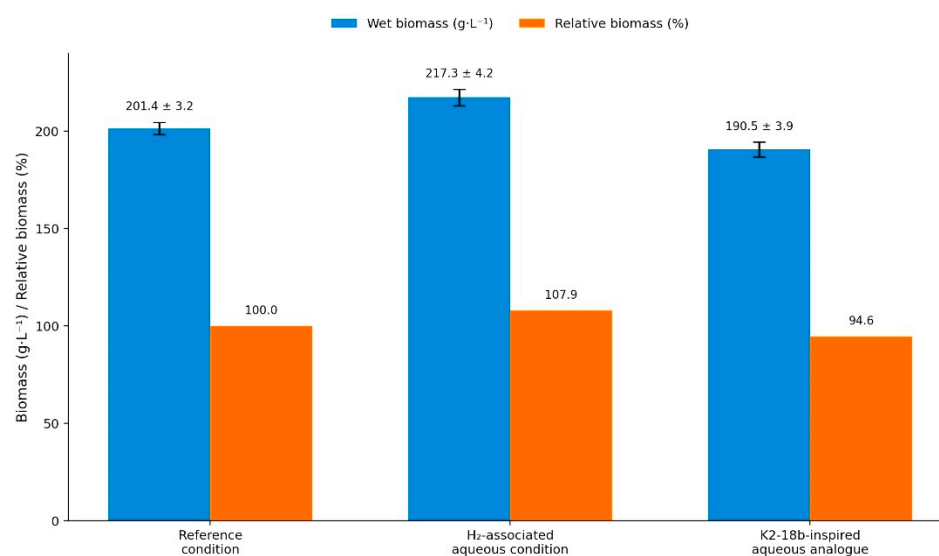


Figure 2. The H₂-associated M9 condition showed numerically shorter lag and generation times and a 7.9% higher wet biomass relative to the reference condition.

The strain retained measurable growth under all investigated conditions. Enrichment of the M9 medium with dissolved H₂ was associated with a shorter lag phase, a slightly reduced generation time, and a 7.9% increase in wet biomass relative to the reference condition.

The individual wet-biomass measurements from three independent biological experiments and corresponding statistical parameters are presented in Table 4.

Table 4. Individual wet-biomass measurements and statistical parameters of *Brevibacterium methyl-icum* B-5652 under reference and H₂-enriched M9 conditions ($n = 3$).

Biological Replicate	Control g·L ⁻¹	H ₂ -Enriched g·L ⁻¹
1	198.4	213.2
2	201.5	217.3
3	204.2	221.4
Mean ± SD	201.4 ± 2.9	217.3 ± 4.1

Wet biomass increased by 7.9% under the H₂-associated condition compared with the reference (217.3 ± 4.1 vs. 201.4 ± 2.9 g·L⁻¹; $n = 3$). Welch's two-sided *t*-test indicated a statistically significant difference ($p = 0.00722$), with a large effect size (Hedges' $g = 3.59$).

Transfer to the H₂-enriched bicarbonate–mineral medium resulted in a modest reduction in biomass to 94.6% of the reference value. The absolute difference in wet biomass was 15.9 g·L⁻¹. One-way ANOVA indicated that the experimental conditions significantly affected maximum OD₆₀₀ and wet-biomass accumulation, whereas differences in generation time were less pronounced. These results indicate that the most pronounced response among the measured growth parameters was observed in cumulative biomass formation, while growth was maintained under all three conditions.

3.5. Relationship Between Biomass Formation and L-Phenylalanine Biosynthesis

L-Phenylalanine production generally followed the pattern of wet-biomass formation. The reference culture produced an L-phenylalanine concentration of 0.86 g·L⁻¹, corresponding to a specific yield of 4.27 mg·g⁻¹ wet biomass. In the H₂-enriched M9 medium, the concentration increased to 0.95 g·L⁻¹, with a specific yield of 4.37 mg·g⁻¹. Under the planetary aqueous analogue, the L-phenylalanine concentration was 0.78 g·L⁻¹, corresponding to specific yields of 4.09 mg·g⁻¹ wet biomass (Table 5). These results show that the H₂-associated M9 condition exhibited higher biomass and L-phenylalanine concentration than the reference, whereas the LHS 1140 b-inspired bicarbonate–mineral condition retained measurable biomass formation and L-phenylalanine biosynthesis.

Table 5. The relationship between wet biomass, L-phenylalanine concentration, and specific yield is summarized for the three experimental conditions.

Experimental Conditions	Wet Biomass g·L ⁻¹	L-Phenylalanine g·L ⁻¹	Specific Yield, mg·g ⁻¹ , Wet Biomass
Reference conditions	201.4	0.86	4.27
H ₂ -associated condition	217.3	0.95	4.37
Planetary aqueous analogue	190.5	0.78	4.09

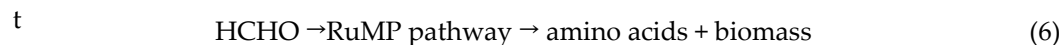
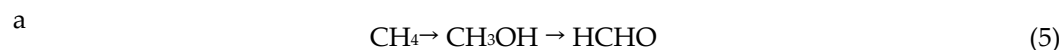
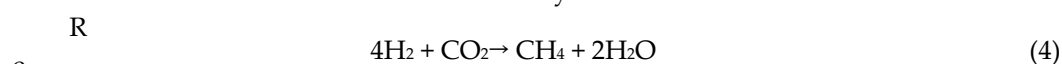
The specific yield was calculated as:

$$Y = \frac{C_{L-phe}}{X_{wetbiomass}} \times 1000 \quad (3)$$

C_{L-Phe} is the concentration of L-phenylalanine and X is the concentration of wet-biomass.

3.6. Conceptual Relationship Between Mineral–Water H_2 Generation and Microbial C_1 Metabolism

The combined physicochemical and microbiological findings can be placed within a conceptual framework linking localized mineral–water H_2 generation with downstream microbial C_1 assimilation and amino-acid biosynthesis:



Reactions (4) and (5) represent literature-supported conceptual steps and were not directly measured in the present experiments; the experimentally investigated microbial segment begins with methanol utilization.

The first stage represents the thermodynamically favorable reduction of CO_2 by molecular hydrogen under reducing planetary conditions. The resulting methane may subsequently undergo partial oxidation to methanol and formaldehyde through photochemical, catalytic, or methanotrophic processes. These conversion steps are treated here as components of the proposed planetary C_1 network rather than as reactions directly mediated by *B. methylicum* B-5652.

The experimentally supported role of *B. Methylicum* B-5652 begins with methanol utilization. Methanol is oxidized to formaldehyde, which occupies a central metabolic position between energy-generating oxidation and constructive carbon assimilation. Formaldehyde-derived carbon is incorporated through the ribulose monophosphate pathway and subsequently converted into cellular biomass and amino acids, including phenylalanine, alanine, valine, and leucine/isoleucine.

The experimentally supported microbial segment of the proposed model begins with methanol oxidation to formaldehyde, followed by RuMP-dependent carbon assimilation and the formation of cellular biomass and amino acids [15]. Glycine and alanine produced within such a C_1 -based biosynthetic framework may subsequently participate in post-biosynthetic self-organization in hydrogen-rich mineral water [27]. Figure 3 integrates these two levels by linking microbial C_1 assimilation with the formation of amino-acid-based microscale structures.

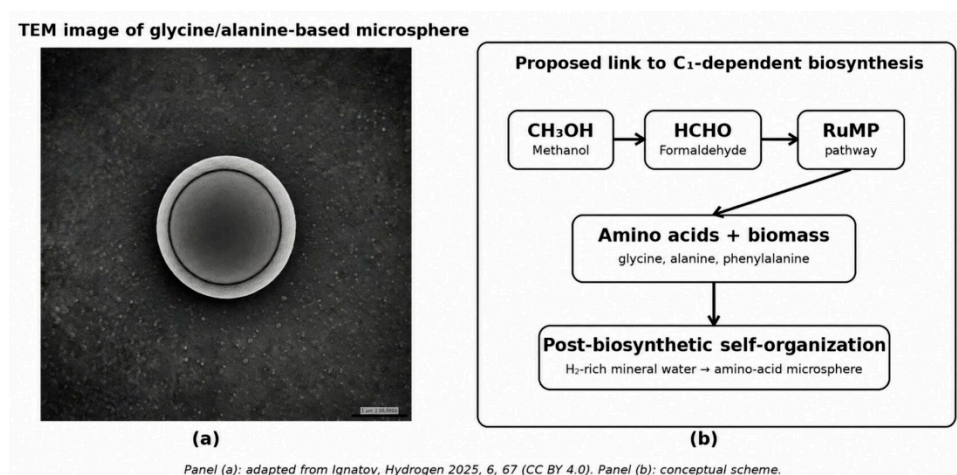


Figure 3. C₁-dependent biosynthesis and microscale organization: (a) adapted glycine/alanine microsphere; (b) RuMP-based pathway. Panel (a) adapted from (Ignatov, Hydrogen 2025), 6, 67 (CC BY 4.0) [27].

Figure 3 extends the planetary-to-microbial model beyond biosynthesis by introducing a possible post-biosynthetic organizational stage. The TEM image demonstrates the experimentally observed formation of a spherical glycine/alanine-based microstructure, whereas the schematic panel places this phenomenon downstream of C₁ assimilation. This relationship should be interpreted as a functional conceptual connection rather than as evidence that *B. methylicum* B-5652 directly produced the observed microsphere.

Accordingly, *B. methylicum* B-5652 represents a terrestrial experimental analogue of the downstream biological segment of a broader planetary C₁-conversion network. Its relevance to LHS 1140 b lies in its demonstrated capacity to transform a reduced one-carbon substrate into structurally complex biological products within an aqueous environment. The model therefore connects atmospheric H₂–CO₂–CH₄ chemistry with methanol-dependent microbial carbon assimilation while preserving a clear distinction between inferred planetary reactions and experimentally verified bacterial metabolism (Figure 4).

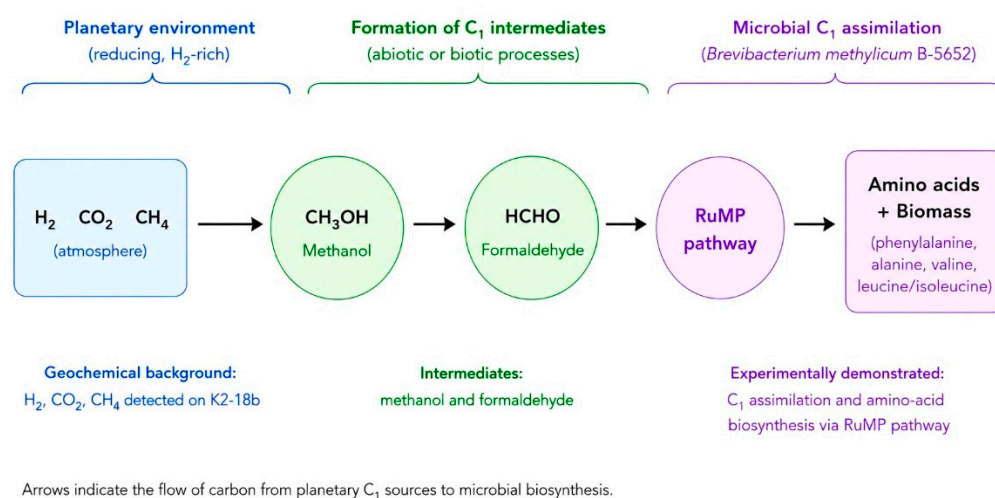


Figure 4. Conceptual planetary-to-microbial C₁ model linking H₂-driven CO₂ reduction and CH₄-derived C₁ intermediates with RuMP-dependent amino-acid biosynthesis and biomass formation in *Brevibacterium methylicum* B-5652. The upstream CO₂-to-CH₄ and CH₄-to-methanol steps were not measured in this study.

Taken together, the results characterize two experimentally distinct levels within the proposed framework: Mg^0 -mediated molecular hydrogen generation and associated aqueous/solid-phase transformations, and methanol-dependent microbial C_1 assimilation with biomass and L-phenylalanine formation. The FT-IR spectrum characterized the hydrated $Mg-O/Mg-OH$ -containing solid products accompanying the reaction, while direct H_2 , ORP, pH, and conductivity measurements defined the resulting reducing aqueous environment. Growth, biomass, methanol conversion, and L-phenylalanine production then provided complementary quantitative indicators of the response of *B. Methylicum* B-5652. These experimentally distinct datasets are integrated within a common terrestrial aqueous framework without implying a directly demonstrated continuous reaction pathway from mineral-generated H_2 to microbial C_1 assimilation.

The present experimental design follows the quantitative framework, in which increasing D_2O content was associated with progressively higher deuterium enrichment of phenylalanine and metabolically related amino acids. The results of Mosin and Ignatov demonstrated that isotope incorporation was amino-acid-specific and reflected differences in precursor pathways, exchangeable hydrogen positions, and the contribution of the aqueous medium to cellular biosynthesis [38].

3.7. Qualitative Spot-Growth Response of *Saccharomyces cerevisiae* After Exposure to Hydrogen Water

Spot growth after 30 min exposure to neutral hydrogen water containing approximately 1.0 ppm dissolved H_2 was qualitatively comparable to that observed for the untreated and distilled-water controls (Figure 5). No obvious reduction in post-exposure growth was observed in any of the four used strains. Similar responses were obtained for the respiration-competent 551 ρ^+ strain, the respiration-deficient 551 ρ^- strain, and the oxidative-stress-sensitive 551 *yap1* Δ strain. Under the conditions of this qualitative assay, hydrogen water produced no evident acute growth-inhibitory effect. The assay did not provide a quantitative measure of cell viability or oxidative stress. The preserved growth of the 551 *yap1* Δ strain, which has impaired YAP1-dependent oxidative-stress regulation, also provided no evidence of pronounced oxidative-stress-associated growth impairment during the 30 min exposure.

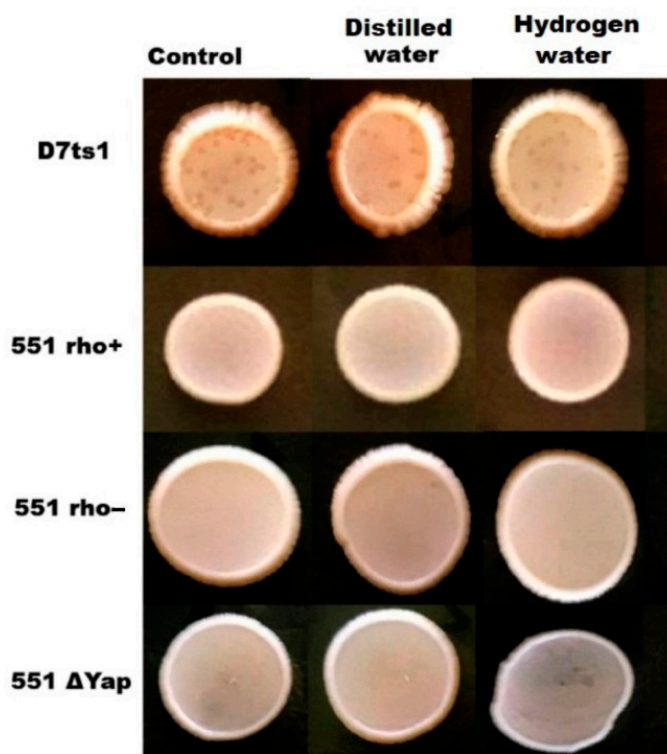


Figure 5. Comparable spot growth of four *Saccharomyces cerevisiae* strains after 30 min exposure to hydrogen water containing approximately 1.0 ppm dissolved H_2 , relative to untreated and distilled-water controls.

Previous studies demonstrated that *S. cerevisiae* and *E. coli* can survive and proliferate in liquid cultures maintained under a 100% H_2 atmosphere [49]. The present experiment examined a complementary aqueous condition in which four *S. cerevisiae* strains were exposed for 30 min to H_2 -containing water with an initial dissolved- H_2 concentration of approximately 1.0 ppm. Comparable spot intensities between the treated cells and the controls indicated no obvious reduction in post-exposure growth under the applied conditions. The similar responses of the respiration-competent 551 ρ^+ and respiration-deficient 551 ρ^- strains suggest that mitochondrial respiratory competence did not markedly influence short-term tolerance to the hydrogen-enriched aqueous phase. Moreover, the preserved growth of the oxidative-stress-sensitive 551 $yap1\Delta$ strain, which has impaired YAP1-dependent stress regulation, provided no evidence of a pronounced pro-oxidative challenge. Collectively, these observations indicate no obvious acute growth-inhibitory effect under the tested 30 min exposure conditions but do not demonstrate H_2 -dependent yeast metabolism or long-term physiological adaptation.

The present study develops an LHS 1140 b-inspired aqueous experimental framework in which mineral–water reactions provide a localized source of molecular H_2 and reducing conditions. The study combines physicochemical characterization of H_2 generation with microbial measurements using *B. methylicum* and complementary short-term assays with *S. cerevisiae*. The objective is not to reproduce the complete atmospheric or oceanic environment of LHS 1140 b, which remains observationally uncertain, but to test a more specific and experimentally accessible hypothesis: whether locally generated dissolved H_2 at a mineral–water interface can modify microbial growth, biomass formation, or short-term cellular response in a water-rich planetary analogue system. In this framework, LHS 1140 b provides the principal water-world context, whereas K2-18

b serves as a complementary example demonstrating the broader diversity of H₂-bearing and volatile-rich environments among temperate exoplanets.

In the present study, the Gram-positive aerobic facultative methylotroph *Brevibacterium methylicum* B-5652 was selected as an experimental astrobiological analogue because it can grow on methanol as a C₁ carbon and energy source and assimilate formaldehyde-derived carbon through the ribulose monophosphate pathway [15]. This metabolic organization provides a biologically grounded terrestrial test system for examining microbial growth and biosynthetic activity under mineral–water H₂-generating and LHS 1140 b-inspired bicarbonate–mineral aqueous conditions. The present results support the use of reactive mineral–water interfaces as experimentally accessible systems for studying localized reducing aqueous environments. Laboratory simulations of alkaline hydrothermal systems have demonstrated that mineral surfaces and geochemical gradients can provide spatially heterogeneous reaction environments relevant to prebiotic chemistry [66,67]. In the present Mg⁰–water system, FT-IR evidence for hydrated and hydroxylated Mg-containing products is consistent with previous vibrational and theoretical studies showing strong water adsorption, surface hydroxylation, and hydration of MgO/Mg-containing surfaces [68,69]. Carbonate-containing Mg phases may additionally form through interaction of alkaline Mg phases with CO₂, as demonstrated for hydrous carbonate-containing brucite systems [60].

The possible chemical relevance of such Mg-bearing phases extends beyond H₂ generation. Chimiak et al. Demonstrated experimentally that ferroan brucite can mediate glycine synthesis from nitrate and glyoxylate, directly linking brucite-type mineral phases with prebiotic amino-acid chemistry [65]. These observations support the broader interpretation that Mg-containing mineral–water interfaces may provide chemically active microsites rather than functioning only as passive solid surfaces. Experimental studies provide terrestrial mineral–water systems relevant to localized H₂-containing aqueous environments. A 2025 study investigated Mg⁰–water interactions as a source of dissolved H₂ and examined the growth responses of *Hydrogenobacter thermophilus* at H₂ concentrations of approximately 1–2 ppm [26]. These findings provide experimental support for studying localized H₂ generation and reducing conditions at mineral–water interfaces as terrestrial analogues of redox-active aqueous microenvironments considered within the present LHS 1140 b-inspired framework [26].

Dissolved H₂ was measured before inoculation but not monitored during cultivation. The observed 7.9% increase in wet biomass represents a condition-dependent biological response and does not establish an H₂-specific effect.

On Earth, microorganisms capable of using molecular H₂ as an electron donor occur in alkaline and submarine hydrothermal systems, geothermal springs, hot mineral waters, anoxic sediments, and subsurface environments. These hydrogenotrophic communities include methanogenic archaea, acetogenic and sulfate-reducing microorganisms, and aerobic or microaerophilic hydrogen-oxidizing bacteria. Such terrestrial ecosystems demonstrate that geologically generated H₂ can sustain microbial energy metabolism under mineral-rich and reducing aqueous conditions [12,19,26].

The biological results should, however, be interpreted at the level directly tested experimentally. *Brevibacterium methylicum* B-5652 was used as a terrestrial aerobic, facultatively methylotrophic test system for downstream C₁ metabolism. The experimentally supported microbial segment begins with methanol oxidation to formaldehyde, followed by RuMP-dependent carbon assimilation, biomass formation, and L-phenylalanine biosynthesis. The higher wet biomass observed in the H₂-associated M9 condition therefore represents a condition-dependent biological response, but it should not be attributed exclusively to a constant dissolved-H₂ concentration throughout cultivation. An H₂-specific effect cannot be established because dissolved H₂ was not moni-

tored during cultivation. The three independent experiments provide valuable evidence of this response, although their limited number warrants cautious interpretation and further confirmation.

The energy metabolism of this bacterial species, however, is associated with aerobic oxidation and electron transport to O_2 with oxidative phosphorylation. When grown on methanol, the strain extracts energy by aerobic oxidation of the C_1 substrate and subsequent transfer of reducing equivalents to the respiratory electron transport chain, in which molecular O_2 is the final electron acceptor. Energy is conserved by oxidative phosphorylation. Methanol serves as a source of carbon and energy. Its first oxidation to formaldehyde is mediated by NAD^+ -dependent methanol dehydrogenase. Formaldehyde can be directed to two functionally related processes: part of the C_1 flux is assimilated via the RuMP cycle, and the oxidative flux continues to more oxidized C_1 compounds and generates reducing equivalents that power respiration [15,28]. *S. cerevisiae* lacks a classical proton-pumping mitochondrial complex I. NADH is supplied to ubiquinone by the alternative NADH dehydrogenases Ndi1/Nde1/Nde2, after which electrons pass through complex III $\rightarrow$ cytochrome c $\rightarrow$ complex IV $\rightarrow O_2$, and the proton gradient drives ATP synthase [69].

Because the present yeast experiments assess short-duration exposure rather than sustained growth under an exoplanetary atmosphere, they are interpreted specifically as a test of cellular tolerance to the selected aqueous H_2 conditions and not as evidence that terrestrial yeasts could inhabit LHS 1140 b [8,17]. This distinction allows experimentally observed biological effects to be separated from broader assumptions concerning the unknown biology or atmospheric oxygen content of the planet [8,11].

At the planetary level, K2-18 b provides a useful comparative example of how volatile-rich and potentially hydrothermal sub-Neptune environments may couple deep aqueous geochemistry with atmospheric composition [70]. Martin et al. further reviewed the close correspondence between the chemistry of the hydrothermal H_2 – CO_2 redox couple and the central energy-yielding reactions of contemporary anaerobic autotrophic microorganisms [12]. Hydrothermal systems combine a continuous supply of reducing compounds, catalytic mineral surfaces, thermal gradients, pH differences, and microporous spaces. This combination has led to the proposal that such environments could have acted as natural reaction systems, facilitating transitions from geochemistry toward early biochemical organization [12]. However, interior models remain strongly dependent on whether such planets contain deep water oceans, high-pressure ice, or magma-dominated layers [71]. Accordingly, these K2-18 b studies are used here only as comparative examples of H_2 -rich planetary chemistry, whereas LHS 1140 b provides the principal water-world-inspired context for the present mineral–water framework. The main contribution of this study is therefore the experimental integration of mineral–water H_2 generation and solid-phase transformation with independently measured microbial biomass formation and methanol-dependent RuMP-associated biosynthesis.

4. Conclusions

This study establishes an integrated terrestrial experimental framework combining Mg^0 -mediated molecular hydrogen generation, mineral–water transformation, and microbial C_1 -dependent biosynthetic activity within an LHS 1140 b-inspired aqueous context. Interaction of zero-valent magnesium with water generated an H_2 -containing reducing aqueous phase and hydrated/hydroxylated Mg – O / Mg – OH -containing solid products, demonstrating the capacity of reactive mineral–water interfaces to produce localized physicochemical conditions relevant to water-rich planetary environments.

The aerobic, facultatively methylotrophic bacterium *Brevibacterium methylicum* B-5652 remained metabolically active under all investigated conditions. The H_2 -associated

M9 condition produced the highest wet biomass, $217.3 \pm 4.1 \text{ g}\cdot\text{L}^{-1}$, representing a 7.9% increase relative to the reference value of $201.4 \pm 2.9 \text{ g}\cdot\text{L}^{-1}$, whereas the bicarbonate-mineral condition retained 94.6% of the reference biomass. L-Phenylalanine production confirmed preservation of methanol-dependent RuMP-associated biosynthetic activity. These responses are interpreted as condition-dependent microbial effects within the investigated mineral–water system and are not attributed exclusively to a constant dissolved- H_2 concentration throughout cultivation. They do not establish an H_2 -specific mechanism, because dissolved H_2 was measured before inoculation but not during cultivation.

The principal contribution of this work is therefore the experimental integration of mineral-water H_2 generation with a downstream, independently measurable biological C_1 -assimilation system. The experimentally demonstrated microbial sequence begins with methanol utilization, RuMP-dependent carbon assimilation, biomass formation, and L-phenylalanine biosynthesis; upstream CO_2 reduction, CH_4 formation, and methane-to-methanol conversion remain conceptual or literature-supported components and were not directly measured in the present study. Complementary short-term experiments with *Saccharomyces cerevisiae* further showed no obvious reduction in subsequent growth after exposure to H_2 -containing water.

LHS 1140 b is used here as a water-world-inspired planetary framework rather than as a claim that its actual atmospheric, oceanic, or biological conditions were reproduced experimentally. The results demonstrate that localized mineral-water H_2 -generating systems can be experimentally coupled with microbial growth and C_1 -dependent biosynthetic processes, providing a testable platform for future studies of redox-active aqueous microenvironments relevant to water-rich planetary habitability.

This publication is dedicated to the 60th anniversary of the birth and the 10th anniversary of the passing of Assoc. Prof. Oleg Mosin (1966–2016). It is based on experiments he designed and conducted, for which we express our heartfelt gratitude.

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References

1. Madhusudhan, N.; Sarkar, S.; Constantinou, S.; Holmberg, M.; Piette, A.A.A.; Moses, J.I. Carbon-Bearing Molecules in a Possible Hycean Atmosphere. *Astrophys. J. Lett.* **2023**, *956*, L13.
2. Holmberg, M.; Madhusudhan, N. Possible hycean conditions in the sub-Neptune TOI-270 d. *Astron. Astrophys.* **2024**, *683*, L2.
3. Mikal-Evans, T.; Madhusudhan, N.; Dittmann, J.; Günther, M.N.; Welbanks, L.; Van Eylen, V.; Crossfield, I.J.M.; Daylan, T.; Kreidberg, L. Hubble Space Telescope transmission spectroscopy for the temperate sub-Neptune TOI-270 d: A possible hydrogen-rich atmosphere containing water vapor. *Astron. J.* **2023**, *165*, 84.
4. Allart, R.; Bourrier, V.; Lovis, C.; Ehrenreich, D.; Spake, J.J.; Wyttenbach, A.; Pino, L.; Pepe, F.; Sing, D.K.; Lecavelier des Etangs, A. Spectrally resolved Helium absorption from the extended atmosphere of a warm Neptune-mass exoplanet. *Science* **2018**, *362*, 1384–1387.

5. Ehrenreich, D.; Bourrier, V.; Wheatley, P.J.; Lecavelier des Etangs, A.; Hébrard, G.; Udry, S.; Bonfils, X.; Delfosse, X.; Désert, J.-M.; Sing, D.K.; et al. A Giant comet-like cloud of Hydrogen escaping the warm Neptune-mass exoplanet GJ 436b. *Nature* **2015**, *522*, 459–461.
6. Schlawin, E.; Ohno, K.; Bell, T.J.; Murphy, M.M.; Welbanks, L.; Beatty, T.G.; Greene, T.P.; Fortney, J.J.; Parmentier, V.; Edelman, I.R.; et al. Possible Carbon Dioxide above the thick aerosols of GJ 1214 b. *Astrophys. J. Lett.* **2024**, *974*, L33.
7. Wogan, N.F.; Batalha, N.E.; Zahnle, K.J.; Krissansen-Totton, J.; Tsai, S.-M.; Hu, R. JWST Observations of K2-18b Can Be Explained by a Gas-Rich Mini-Neptune with No Habitable Surface. *Astrophys. J. Lett.* **2024**, *963*, L7.
8. Damiano, M.; Bello-Arufe, A.; Yang, J.; Hu, R. LHS 1140 b is a potentially habitable water world. *Astrophys. J. Lett.* **2024**, *968*, L22.
9. Mayhew, L.E.; Ellison, E.T.; McCollom, T.M.; Trainor, T.P.; Templeton, A.S. Hydrogen generation from low-temperature water–rock reactions. *Nat. Geosci.* **2013**, *6*, 478–484.
10. Schmidt, S.P.; MacDonald, R.J.; Tsai, S.-M.; Radica, M.; Wang, L.C.; Ahrer, E.-M.; Bell, T.J.; Fisher, C.; Thorngren, D.P.; Wogan, N.; et al. A Comprehensive reanalysis of K2-18 b’s JWST NIRISS + NIRSpec Transmission Spectrum. *Astron. J.* **2025**, *170*, 298.
11. Cadieux, C.; Plotnykov, M.; Doyon, R.; Valencia, D.; Jahandar, F.; Dang, L.; Turbet, M.; Fauchez, T.J.; Cloutier, R.; Cherubim, C.; et al. New mass and radius constraints on the LHS 1140 planets: LHS 1140 b Is either a temperate mini-Neptune or a water world. *Astrophys. J. Lett.* **2024**, *960*, L3.
12. Martin, W.; Baross, J.; Kelley, D.; Russell, M.J. Hydrothermal vents and the origin of life. *Nat. Rev. Microbiol.* **2008**, *6*, 805–814.
13. Lollar, B.S.; Onstott, T.C.; Lacrampe-Couloume, G.; Ballentine, C.J. The contribution of the Precambrian continental lithosphere to global H₂ production. *Nature* **2014**, *516*, 379–382.
14. Preiner, M.; Igarashi, K.; Muchowska, K.B.; Yu, M.; Varma, S.J.; Kleinermanns, K.; Nobu, M.K.; Kamagata, Y.; Tüysüz, H.; Moran, J.; et al. A hydrogen-dependent geochemical analogue of primordial carbon and energy metabolism. *Nat. Ecol. Evol.* **2020**, *4*, 534–542.
15. Mosin, O.V.; Skladnev, D.A.; Shvets, V.I. Biosynthesis of ²H-Labeled Phenylalanine by a New Methylophilic Mutant *Brevibacterium methylcum*. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 225–229.
16. Mosin, O.V.; Shvets, V.I.; Skladnev, D.A.; Ignatov, I. Microbial synthesis of ²H-labelled L-phenylalanine with different levels of isotopic enrichment by a facultative methylophilic bacterium *Brevibacterium methylcum* with RuMP assimilation of carbon. *Biochem. (Mosc.) Suppl. Ser. B Biomed. Chem.* **2013**, *7*, 247–258.
17. Seager, S.; Petkowski, J.J.; Günther, M.N.; Bains, W.; Mikal-Evans, T.; Deming, D. Possibilities for an aerial biosphere in temperate sub Neptune-sized exoplanet atmospheres. *Universe* **2021**, *7*, 172.
18. Glein, C.R. The geochemical potential for metabolic processes on the sub-Neptune exoplanet K2-18b. *Astrophys. J. Lett.* **2024**, *964*, L19.
19. Martin, W.; Russell, M.J. On the origin of biochemistry at an alkaline hydrothermal vent. *Philos. Trans. R. Soc. B Biol. Sci.* **2007**, *362*, 1887–1926.
20. Lane, N.; Martin, W.F. The origin of membrane bioenergetics. *Cell* **2012**, *151*, 1406–1416.
21. Szostak, J.W. On the origin of life. *Medicina* **2016**, *76*, 199–203.
22. Damer, B.; Deamer, D. The hot spring hypothesis for an origin of life. *Astrobiology* **2020**, *20*, 429–452.
23. Miller, S.L.; Urey, H.C. Organic compounds synthesis on the primitive Earth: Several questions about the origin of life have been answered, but much remains to be studied. *Science* **1959**, *130*, 245–251.
24. Wilson, A.T. Synthesis of macromolecules under possible primeval earth conditions. *Nature* **1960**, *188*, 1007–1009.
25. Ignatov, I.I.; Popova, T.P. Possible approaches for the origin of life in hot mineral water in primary hydrosphere. *Adv. Life Sci.* **2025**, *12*, 399–412.
26. Ignatov, I.; Popova, T.P.; Vassileva, P.; Marinov, Y.G.; Iliev, M.T. Hot mineral water as a medium for molecular hydrogen reactions in the primordial hydrosphere for the origin of life. *Hydrogen* **2025**, *6*, 48.
27. Ignatov, I. Microstructures as models for origin of life in hot water: Hydrogen-assisted self-assembly of glycine and alanine zwitterions. *Hydrogen* **2025**, *6*, 67.
28. Chistoserdova, L.; Kalyuzhnaya, M.G.; Lidstrom, M.E. The expanding world of methylophilic metabolism. *Annu. Rev. Microbiol.* **2009**, *63*, 477–499.
29. Klein, V.J.; Irla, M.; Gil López, M.; Brautaset, T.; Fernandes Brito, L. Unravelling formaldehyde metabolism in bacteria: Road towards synthetic methylophilicity. *Microorganisms* **2022**, *10*, 220.
30. Artuso, I.; Turrini, P.; Pirolo, M.; Lugli, G.A.; Ventura, M.; Visca, P. Phylogenomic reconstruction and metabolic potential of the genus *Aminobacter*. *Microorganisms* **2021**, *9*, 1332.

31. Jeon, Y.C.; Nguyen, A.D.; Lee, E.Y. Bioproduction of isoprenoids and other secondary metabolites using methanotrophic bacteria as an alternative microbial cell factory option: Current stage and future aspects. *Catalysts* **2019**, *9*, 883.
32. Iguchi, H.; Yurimoto, H.; Sakai, Y. Interactions of methylotrophs with plants and other heterotrophic bacteria. *Microorganisms* **2015**, *3*, 137–151.
33. Yurimoto, H.; Shiraishi, K.; Sakai, Y. Physiology of methylotrophs living in the Phyllosphere. *Microorganisms* **2021**, *9*, 809.
34. Lin, N.; Tao, Y.; Gao, P.; Xu, Y.; Xing, P. Comparative genomics revealing insights into Niche separation of the Genus *Methylophilus*. *Microorganisms* **2021**, *9*, 1577.
35. Islam, T.; Larsen, Ø.; Torsvik, V.; Øvreås, L.; Panosyan, H.; Murrell, J.C.; Birkeland, N.-K.; Bodrossy, L. Novel methanotrophs of the family Methylococcaceae from different geographical regions and habitats. *Microorganisms* **2015**, *3*, 484–499.
36. Mosin, O.V.; Shvets, V.I.; Skladnev, D.A.; Ignatov, I. Studying of microbic synthesis of deuterium labelled L-Phenylalanin by facultative methylotrophic bacterium *Brevibacteriummethylicum* on media with different content of heavy water. *Russ. J. Biopharm.* **2012**, *4*, 11–22.
37. Skladnev, D.A.; Mosin, O.V.; Egorova, T.A.; Eremin, S.V.; Shvets, V.I. Methylotrophic bacteria as sources of 2H-and 13C-amino Acids. *Biotechnology* **1996**, *5*, 14–22.
38. Mosin, O.V.; Ignatov, I. Microbiological synthesis of 2H-labeled Phenylalanine, Alanine, Valine, and Leucine/Isoleucine with different degrees of Deuterium enrichment by the Gram-positive facultative methylotrophic bacterium *Brevibacteriummethylicum*. *Int. J. Biomed.* **2013**, *3*, 132–138.
39. Sasselov, D.D.; Grotzinger, J.P.; Sutherland, J.D. The origin of life as a planetary phenomenon. *Sci. Adv.* **2020**, *6*, eaax3419.
40. Hazen, R.M.; Sverjensky, D.A. Mineral surfaces, geochemical complexities, and the origins of life. *Cold Spring Harb. Perspect. Biol.* **2010**, *2*, a002162.
41. Russell, M.J. Green Rust: The Simple Organizing ‘Seed’ of All Life? *Life* **2018**, *8*, 35.
42. Cleaves, H.J., II; Michalkova Scott, A.; Hill, F.C.; Leszczynski, J.; Sahai, N.; Hazen, R. Mineral–organic interfacial processes: Potential roles in the origins of life. *Chem. Soc. Rev.* **2012**, *41*, 5502–5525.
43. Singh, H.B.; Kang, M.-K.; Kwon, M.; Kim, S.-W. Developing methylotrophic microbial platforms for a methanol-based bioindustry. *Front. Bioeng. Biotechnol.* **2022**, *10*, 1050740.
44. Todorova, A.; Todorova, T. Apricot kernels’ extract and amygdalin alter bleomycin-induced Ty1 retrotransposition, mitotic gene conversion in the trp-5 locus and reverse point mutations in ilv1-92 allele in *Saccharomyces cerevisiae*. *Arch. Microbiol.* **2022**, *204*, 542.
45. Kuzucan, A.; Bolmont, E.; Chaverot, G.; Ferreira, J.Q.; Ibelings, B.W.; Bhatnagar, S.; McGinnis, D.F. The role of atmospheric composition in defining the habitable zone limits and supporting *E. coli* growth. *Life* **2025**, *15*, 79.
46. Singhopon, T.; Shinoda, K.; Rujakom, S.; Kazama, F. Factors affecting the simultaneous removal of nitrate and reactive black 5 dye via hydrogen-based denitrification. *Water* **2021**, *13*, 922.
47. Xiao, L.; Miwa, N. Hydrogen-Rich Water Prevents dehydration-induced cellular oxidative stress and cell death in human skin keratinocytes. *Hydrogen* **2022**, *3*, 62–71.
48. Nakao, A.; Toyoda, Y.; Sharma, P.; Evans, M.; Guthrie, N. Effectiveness of hydrogen rich water on antioxidant status of subjects with potential metabolic syndrome—An open label pilot study. *J. Clin. Biochem. Nutr.* **2010**, *46*, 140–149.
49. Vassilev, N.; Ignatov, I.; Popova, T.P.; Huether, F.; Ignatov, A.I.; Iliev, M.T.; Marinov, Y. Nuclear Magnetic Resonance (NMR) and Density Functional Theory (DFT) Study of water clusters of Hydrogen-rich water (HRW). *Water* **2024**, *16*, 3261.
50. Slavov, D.; Tomaszewska, E.; Grobelny, J.; Drenchev, N.; Karashanova, D.; Peshev, Z.; Bliznakova, I. FTIR spectroscopy revealed nonplanar conformers, chain order, and packaging density in diOctadecylamine- and octadecylamine-passivated gold nanoparticles. *J. Mol. Struct.* **2024**, *1314*, 138827.
51. Ignatov, I.; Marinov, Y.G.; Vassileva, P.; Gluhchev, G.; Pesotskaya, L.A.; Jordanov, I.P.; Iliev, M.T. Nonlinear hydrogen bond network in small water clusters: Combining NMR, DFT, FT-IR, and EIS research. *Symmetry* **2025**, *17*, 1062.
52. Studier, F.W. Protein production by auto-induction in high-density shaking cultures. *Protein Expr. Purif.* **2005**, *41*, 207–234.
53. Zwietering, M.H.; Jongenburger, I.; Rombouts, F.M.; Van’t Riet, K. Modeling of the bacterial growth curve. *Appl. Environ. Microbiol.* **1990**, *56*, 1875–1881.
54. Todorova, T.; Boyadzhiev, K.; Dimitrov, M.; Parvanova, P. Bee venom genotoxicity on *Saccharomyces cerevisiae* cells—The role of mitochondria and YAP1 transcription factor. *Toxicology* **2024**, *503*, 153768.
55. Zarev, Y.; Nedelcheva, R.; Todorova, T.; Boyadzhiev, K.; Dimitrov, M.; Ionkova, I. Discovery of novel compounds from *Li-numleonii* hairy roots with anticarcinogenic and cytoprotective potential. *Rev. Bras. Farmacogn.* **2025**, *35*, 798–809.

56. Todorova, T.; Boydzhiiev, K.; Ignatov, I.; Petrova Popova, T.; Dimitrov, Z.; Gotova, I.; Huether, F.; Ignatov, A.I.; Georgiev Marinov, Y. L-Malic acid descender for drinking water—Physicochemical analysis and biological activity. *Beverages* **2025**, *11*, 62.
57. Sagadevan, S.; Lett, J.A.; Fatimah, I.; Selvi, K.T.; Sivasankaran, R.P.; Weldegebrerial, G.K.; Oh, W.-C. Photocatalytic and electrochemical activity of magnesium oxide nanoballs synthesized via a hydrothermal route. *Processes* **2022**, *10*, 2098.
58. Piperopoulos, E.; Fazio, M.; Mastronardo, E.; Lanza, M.; Milone, C. Tuning Mg(OH)₂ structural, physical, and morphological characteristics for its optimal behavior in a thermochemical heat-storage application. *Materials* **2021**, *14*, 1091.
59. Ignatov, I.; Marinov, Y.G.; Vassileva, P.; Popova, T.P.; Gluhchev, G.; Iliev, M.T.; Huether, F.; Dimitrov, Z.; Gotova, I. Formation of ice I_h clusters in solid-phase glacial water with low concentrations of Ca²⁺ and Mg²⁺ ions. *Crystals* **2025**, *15*, 254.
60. German, A.; Winnefeld, F.; Lura, P.; Rentsch, D.; Lothenbach, B. Hydrous carbonate-containing brucite (HCB) in MgO/hydromagnesite blends. *Cem. Concr. Res.* **2023**, *173*, 107304.
61. Chen, J.; Huang, L.; Dong, L.; Zhang, H.; Huang, Z.; Li, F.; Zhang, S. Hydration behavior of MgO surface: A first-principles study. *Appl. Surf. Sci.* **2023**, *611*, 155441.
62. Li, B.; Chen, H.; Feng, J.; Ma, Q.; Chen, J.; Ren, B.; Yin, S.; Jiang, P. First principles calculation of adsorption of water on MgO (100) plane. *Materials* **2023**, *16*, 2100.
63. García-Hernández, C.M.; López-Cuevas, J.; Gutiérrez-Chavarría, C.A.; Flores-Valdés, A. Use of mechanical activation to obtain Mg(OH)₂ from olivine mineral for CO₂ capture. *Bol. Soc. Esp. Ceram. Vidr.* **2021**, *60*, 163–174.
64. Wu, S.; Tan, B.T.; Senevirathna, H.L.; Wu, P. Polarization of CO₂ for improved CO₂ adsorption by MgO and Mg(OH)₂. *Appl. Surf. Sci.* **2021**, *562*, 150187.
65. Chimiak, L.; Hara, E.; Sessions, A.; Templeton, A.S. Glycine synthesis from Nitrate and Glyoxylate mediated by Ferroan Brucite: An integrated pathway for prebiotic amine synthesis. *Proc. Natl. Acad. Sci. USA* **2024**, *121*, e2408248121.
66. Herschy, B.; Whicher, A.; Camprubí, E.; Watson, C.; Dartnell, L.; Ward, J.; Evans, J.R.G.; Lane, N. An origin-of-life reactor to simulate alkaline hydrothermal vents. *J. Mol. Evol.* **2014**, *79*, 213–227.
67. Westall, F.; Hickman-Lewis, K.; Hinman, N.; Gautret, P.; Campbell, K.A.; Bréhéret, J.-G.; Foucher, F.; Hubert, A.; Sorieul, S.; Dass, A.V.; et al. A Hydrothermal-sedimentary context for the origin of life. *Astrobiology* **2018**, *18*, 259–293.
68. Dekermenjian, M.; Merlen, A.; Ruediger, A.; Rérat, M. Water adsorption on MgO surfaces: A vibrational analysis. *Crystals* **2023**, *13*, 1153.
69. Eldeeb, M.H.; Camacho Lopez, L.J.; Fontanesi, F. Mitochondrial respiratory supercomplexes of the yeast *Saccharomyces cerevisiae*. *IUBMB Life* **2024**, *76*, 485–504.
70. Luu, C.N.; Yu, X.; Glein, C.R.; Innes, H.; Aguiquine, A.; Krissansen-Totton, J.; Moses, J.I.; Tsai, S.-M.; Zhang, X.; Truong, N.; et al. Volatile-rich Sub-Neptunes as hydrothermal worlds: The case of K2-18 b. *Astrophys. J. Lett.* **2024**, *977*, L51.
71. Shorttle, O.; Jordan, S.; Nicholls, H.; Lichtenberg, T.; Bower, D.J. Distinguishing oceans of water from Magma on mini-Neptune K2-18b. *Astrophys. J. Lett.* **2024**, *962*, L8.

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