

Supplementary Information:

BioQuantum Record: Exploring otherness through extremophilic microorganisms

Anna Steward^{1,†}, Sebastian V. Gfeller^{2,†,*}, Carlo Pifferi³, Marie Catherine Sforza², Vincent Aucagne³, Matthieu Réfrégiers³

¹Academy of Fine Arts, Nuremberg, Germany

²UPR4301 Center for Molecular Biophysics (CBM), University of Orléans, Orléans, France

³UPR4301 Center for Molecular Biophysics (CBM), Orléans, France

† = The authors contributed equally to this work.

* = Corresponding author: sebastian.gfeller@cnrs-orleans.fr

Mirror Life – Testing Enzymatic Boundaries

Mirror Life and Chiral Handshake

A scientific question has arisen from an artistic narrative: Can our microbial astronauts carry the proposed sweet chiral gifts to their extra-terrestrial counterparts? Or will they “eat” it during their long interstellar travel, which would be inappropriate, impolite, and diplomatically incorrect? In biomolecules such as amino acids and sugars, chirality arises from the presence of at least one carbon atom that engages in covalent bonds with four different substituents, giving rise to two possible forms, called enantiomers, which are chirally inverted “mirror images” in three dimensions (**Figure 1**).

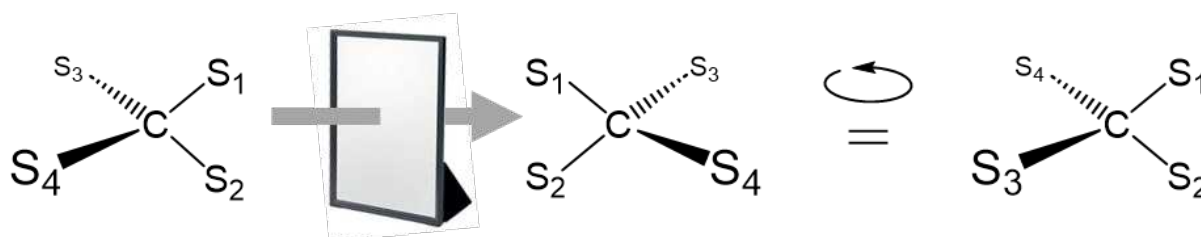


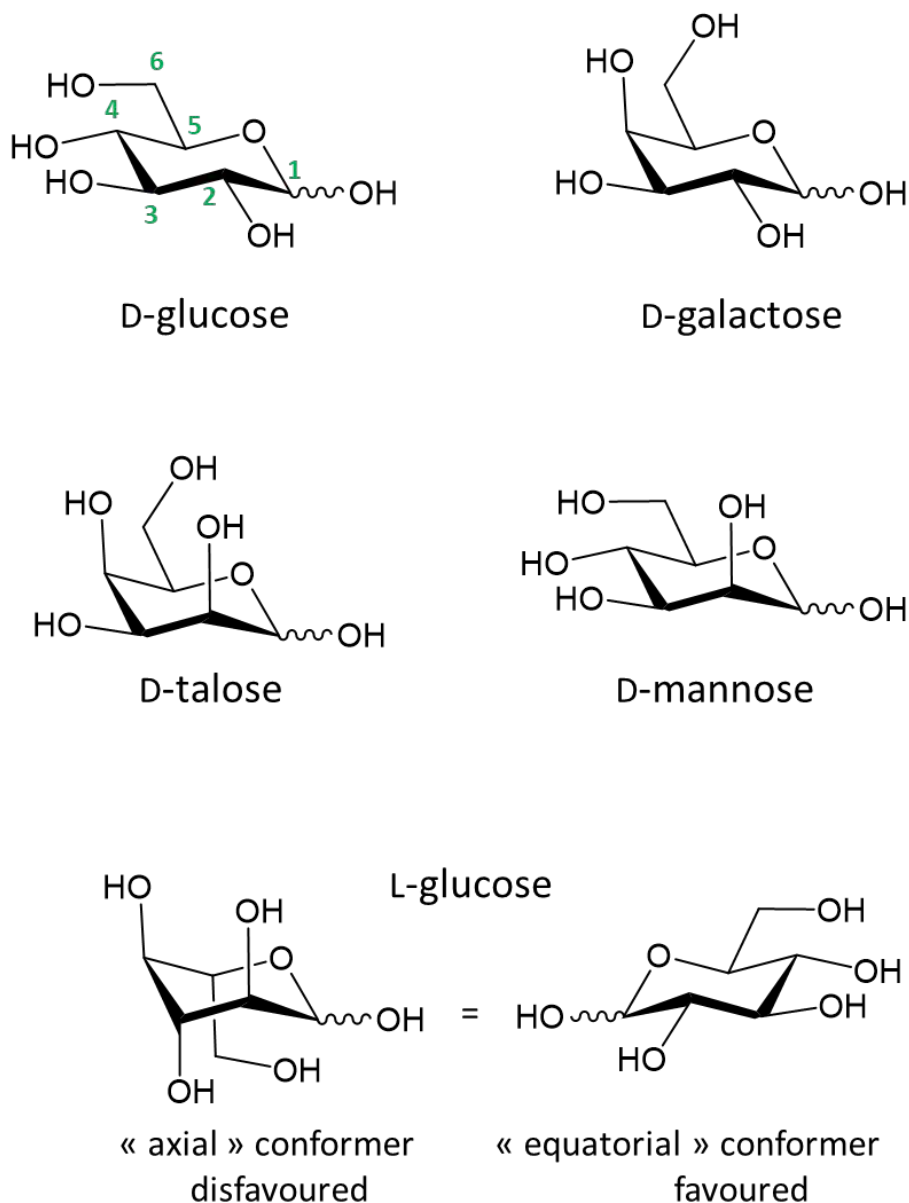
Figure 1: A tetrahedral carbon atom (“C”) with four different substituents, S₁, S₂, S₃, and S₄ (e.g., different groups of atoms), can exist in two different “enantiomeric” chirally inverted forms.

A Chiral Gift – Testing Enzymatic Boundaries

Enzymes are proteins, *i.e.*, macromolecules composed of hundreds of homochiral amino acids linked together and then “folded” into a compact chiral three-dimensional structure capable of recognizing and binding to a “substrate” molecule and catalyzing its transformation into one or several “product” molecules. If the substrate is chiral, the (chiral) enzyme does not operate equally on both its native substrate and its chirally inverted version, such as a left-hand glove that does not fit a right hand. An enzyme selectively interacts with a particular spatial arrangement of atoms in its substrate, typically one enantiomer, while showing little or no activity toward its mirror image (Brik and Wong 2003). This selectivity arises from the three-dimensional complementarity between the active site of the enzyme and the specific configuration of its substrate. As such, even subtle changes (*e.g.*, chirality inversion) can completely abolish binding or catalysis. In biological systems, where molecular recognition and function critically depend on stereochemistry, a chirally inverted substrate is, in most cases, effectively invisible to the enzyme.

In the framework of our art and science project, we focus on enzymes acting on “sugars”, also known as carbohydrates, which are biomolecules typically composed of multiple chiral carbon atoms, each linked to an oxygen atom. Among sugars, in addition to the pentoses (5 carbon atoms) D-2-deoxy-ribose and D-ribose, which are the core chiral parts of DNA and RNA, respectively, the hexose family (6 carbons, 6 oxygens) is the most abundant and includes D-glucose, a crucial source of energy in all organisms on Earth, which is used as a starting substrate for metabolic pathways consisting of cascades of different enzymes. Although the enzymes and reactions they catalyze differ substantially depending on the type of organism, the starting substrate is always conserved: D-glucose.

One would expect an extra-terrestrial form of life to also use glucose as an energy source. Five of the six carbons in glucose are chiral, and other hexoses in which the chirality of one or several carbons is inverted (so-called “diastereoisomers”) play important biological roles. For example, galactose is the carbon 4 (C4) diastereoisomer, mannose is the C1 diastereoisomer, and talose is the C1/C4 (**Figure 2**). A distinctive feature of glucose compared to other hexoses is its ability to adopt a “chair-like” three-dimensional shape (chemists call that a “conformation”), in which the ring core, composed of five carbon and one oxygen atoms, positions its substituents predominantly in equatorial orientations (*i.e.*, lying in the plane defined by the chair). This arrangement makes glucose relatively flat and exceptionally stable, as the bulky oxygen atoms are quite far from each other, avoiding spatial conflicts (“steric clashes”). Such an “all-equatorial” configuration is thought to have been favored by evolution on Earth, making glucose the preferred fuel for life on Earth.



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65 **Figure 2:** Schematic three-dimensional shapes (conformations) of selected hexoses. The
 66 compounds shown are D-glucose, D-galactose, D-talose, D-mannose, and the enantiomer L-
 67 glucose. Each six-atom ring ("pyranose") adopts a chair conformation, highlighting the
 68 differences in the orientation of the hydroxyl groups (axial vs. equatorial) that distinguish the
 69 stereoisomers. In the case of L-galactose, L-mannose, or L-talose, only one or two
 70 substituents are axial, and the chair has the same orientation as L-glucose. In the case of D-
 71 glucose, rotation of the bonds causes it to adopt an inverted chair conformation.

72 Thus, one could imagine that L-glucose, the enantiomer of glucose in which all five
 73 chiral carbons are inverted, could have been selected by homochirality-divergent
 74 earth-independent forms of life as a source of energy because, as a mirror image, it
 75 shares all the stability features of D-glucose, as detailed earlier. L-glucose does not
 76 occur naturally in earth-borne living organisms but can be synthesized in the
 77 laboratory. It adopts an "inverted chair" three-dimensional structure, which is a mirror
 78 image of D-glucose (see **Figure 2**).

In its isolated form, glucose carbon #1 (C1) displays a hydroxyl (-OH) group (in addition to those at C2, C3, C4, and C6) that can rapidly interconvert between two chiral configurations, namely α and β (**Figure 3**); however, the C1 hydroxyl group can also form a covalent bond with another molecule, affording “glycosides” (the hydrogen atom, H, is replaced by another, typically a carbon). As a glycoside, the C1 carbon of glucose is locked in a fixed configuration, either α or β .

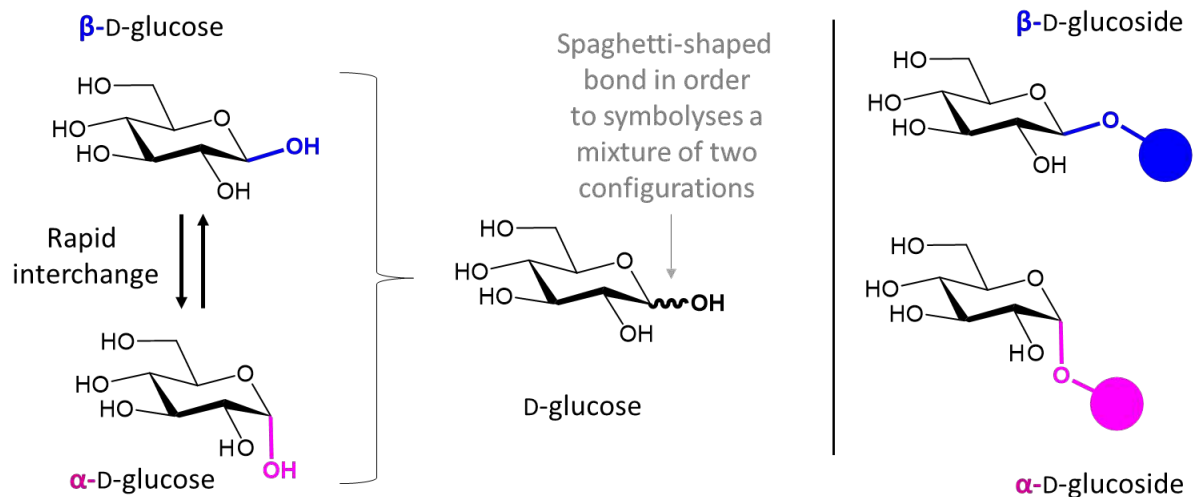


Figure 3: Isolated form of D-glucose, in which the interconversion between its α and β C1 configurations is allowed, versus the glycoside form of D-glucose, in which the C1 chiral configurations are locked.

Many organisms store glucose in polymeric forms, such as glycogen in animals and starch in plants, which can be depolymerized by enzymes on demand to release “free” isolated glucose, which is then used as an energy source. Besides its role in energy storage, cellulose—a glucose polymer composed of linear chains of several hundred to many thousands of β -D-glucose units (**Figure 4**)—is a crucial structural component of plant cell walls, which gives plants strength and rigidity, and is the most abundant organic polymer on Earth: terrestrial plants globally produce ~ 100 billion tonnes of cellulose every year.

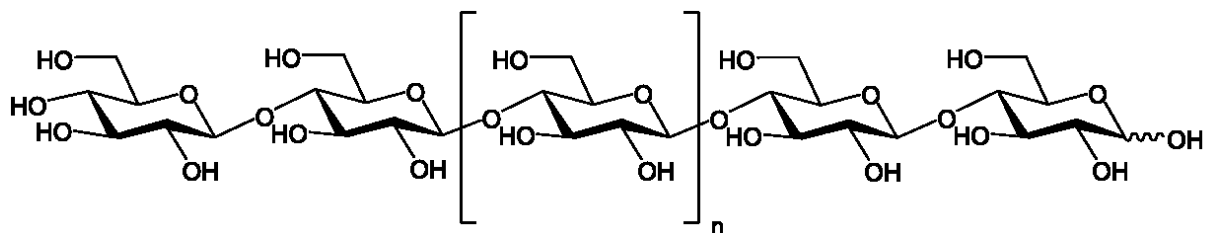


Figure 4: Molecular structure of cellulose.

We focused on β -glucosidases, enzymes that catalyze the breakage of the β -glucosidic bond via the addition of a water molecule (hydrolysis). Although mammals do not produce β -glucosidase enzymes to digest cellulose, they do express certain β -glucosidases for breaking down specific glucosides. However, many microorganisms, including archaea, bacteria (particularly ruminant gut microbiomes), fungi, and

yeasts, express and secrete these enzymes. In our experiment, we investigated whether the enzymes of our chosen microorganism could hydrolyze the β -glucosidic bond in a D-glucoside “molecular probe” compared to an L-glucoside probe. We did not anticipate that Earth-borne enzymes would hydrolyze the mirror-image substrate¹ and aimed to obtain visual feedback on its selectivity. In principle, the mirror-image substrate could only be efficiently hydrolyzed by an “alien” mirror-image glycosidase, thereby serving as precursors of our intended sweet “chiral gift”, L-glucose. Our synthetic sugar probes are chromogenic substrates (*p*-nitrophenyl glucosides) that release a bright yellow compound (*p*-nitrophenolate) upon hydrolysis (**Figure 5**).

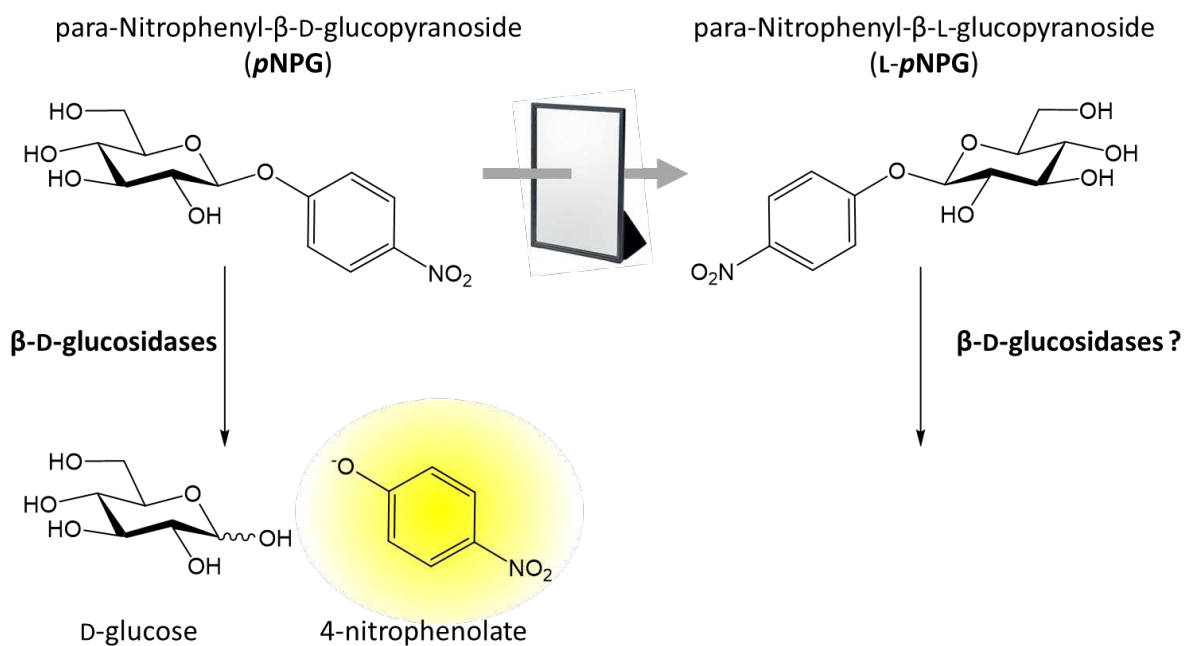


Figure 5: Enzymatic hydrolysis of para-nitrophenyl- β -D-glucopyranoside (*p*NPG) releases bright yellow para-nitrophenolate, whereas the enzyme activity is expected to be null with respect to the L-*p*NPG substrate, which is the mirror-image version of *p*NPG.

The resulting color change provides a straightforward visual signal that can be easily measured; for this reason, these probes are commonly used in laboratory assays to monitor enzyme activity. *p*NPG is commercially available and has been extensively used as a probe to quantify the catalytic efficiency of a purified β -glucosidase enzyme (Riou et al. 1998; Lin et al. 2010) or to detect β -glucosidase secretion by microorganisms such as fungi (Madhu et al. 2009), bacteria (Strahsburger et al. 2017), and yeasts (Liu et al. 2020). To our knowledge, no attempts have been made to monitor β -glucosidase secretion by archaea, although the presence of β -glucosidase has been evidenced (Schröder et al. 2014).

¹ A few microbial enzymes with L-glucosidase activity have been reported. For example, studies have described L-fucosidases and L-rhamnosidases with very weak ability to act on L-glucose linkages, which is considered as promiscuous” catalytic activity, not selected by evolution.

Materials and Methods

Microbial Cultivation

Metallosphaera sedula DSM 5348 was grown aerobically in DSMZ 88 medium with pyrite, as previously described by Gfellner et al. (2025) in 1 L glassblower modified Schott-bottle bioreactors (Duran DWK Life Sciences GmbH, Wertheim/ Main, Germany). The stock culture was stored at -80°C in a solution containing equal parts of 50% glycerol and DSMZ 88 medium (50, 50, v:v). The DSMZ 88 medium contained 9.84 mM $(\text{NH}_4)_2\text{SO}_4$, 2.06 mM KH_2PO_4 , 1.01 mM $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.48 mM $\text{CaCl}_2 \times 2\text{H}_2\text{O}$, and 0.07 mM $\text{FeCl}_3 \times 6\text{H}_2\text{O}$, and it also served as the medium for cell resuspension. Additionally, Allen trace element solution was added, which contained 0.91 mM $\text{MnCl}_2 \times 4\text{H}_2\text{O}$, 1.18 mM $\text{Na}_2\text{B}_4\text{O}_7 \times 10\text{H}_2\text{O}$, 0.08 mM $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$, 0.03 mM $\text{CuCl}_2 \times 2\text{H}_2\text{O}$, 0.01 mM $\text{Na}_2\text{MoO}_4 \times 2\text{H}_2\text{O}$, 0.02 mM $\text{VOSO}_4 \times 2\text{H}_2\text{O}$, and 3.56 μM $\text{CoSO}_4 \times 7\text{H}_2\text{O}$. As previously described by Kölbl et al. (2017), 0.1% tryptone was added to the DSMZ 88 medium. The pH was set to 2.0 using 5 M H_2SO_4 . Pyrite was manually crushed with a hand grinder to achieve particle sizes between 63–100 μm , regulated by 63 and 100 μm mesh sieves, and then baked overnight at 180°C . Pyrite at a concentration of 10 g/L was added to 800 mL of culture. A 1 L bioreactor was assembled, maintained at a constant temperature of 73°C , and continuously stirred. A CO_2 flow at a total rate of 0.9 L/min (normalized to 1 atm and 0°C) was maintained for the interconnected triplicate bioreactor setup, resulting in a flow rate of 0.3 L/min for each bioreactor.

To track microbial growth, samples from the cultures were collected continuously throughout the growth phase, and cell counts were determined using a microscope (Olympus BX51 with a Pixelink M20C-CYL camera) and a Neubauer chamber (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Once the cultures reached the stationary phase, they were collected by centrifugation in sterile 50 mL Falcon tubes at $3220 \times g$ for 40 min.

Furthermore, a 10 \times concentrated *M. sedula* culture grown on pyrite was recultivated in 50 mL Erlenmeyer flasks with the following mineral materials: two Mars analog materials, JEZ-1 Jezero Delta Simulant (Exolith) and ESA01-E Martian basalt analog, pyrite, rutile, anatase, illmenite, and titanium dioxide, in a shake incubator set at 100 rpm and 75°C for 72 h. The harvested material was subsequently transferred in 300 μL steps (3 mL in total) to metal exposure wells and air-dried.

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Halobacterium salinarum DSM 3754 was cultivated aerobically in DSMZ 97 medium² without additional $\text{MnSO}_4 \times \text{H}_2\text{O}$ in 50 mL Erlenmeyer flasks in a shake incubator set at 100 rpm and 37°C for 504 h. The cultures were continuously and directly transferred into 1.5 mL Eppendorf tubes for further experiments. Additionally, a portion of the harvested material was subsequently transferred in 300 μL steps (3 mL in total) to metal exposure wells and air-dried.

Scanning Electron Microscopy

H. salinarum cells were cultivated as described above until they reached a high cell density, which was assessed by light microscopy. A 1 mL aliquot of the culture was fixed by adding glutaraldehyde (Sigma-Aldrich) to a final concentration of 2 %. The samples were fixed for 2.5 h at room temperature.

After fixation, 100 μL of the cell suspension was applied to a Whatman[®] Nuclepore polycarbonate filter disc (0.2 μm pore size, 13 mm diameter; Cytiva). The filters were washed once with double-distilled water and air-dried at room temperature. The dried filters were mounted onto SEM specimen stubs (12.5 mm diameter, 3.2 \times 8 mm pin; Agar Scientific) using double-adhesive tape.

The samples were sputter-coated with gold/palladium (Au/Pd) for 10 s at ~20 mA and 2.45 kV using a Polaron SEM Coating Unit E5100. Scanning electron microscopy (SEM) was performed using a ZEISS GeminiSEM with an in-lens detector operating at an electron high tension (EHT) of 5 kV.

Glucosidase Experiment

para-Nitrophenyl- β -D-glucopyranoside (*p*NPG, CAS RN: 2492-87-7) was purchased from TCI Europe N.V. Its mirror image, *para*-nitrophenyl- β -L-glucopyranoside (*L-p*NPG) has never been described, and was prepared following a three-steps synthetic route described for *p*NPG [Riou et al., 1998], starting from L-glucose (Sigma-Aldrich). Its ¹H NMR spectrum was consistent with the literature data for *p*NPG [Riou et al., 1998] and was identical to that of commercial *p*NPG, as expected for enantiomeric compounds. Similarly, reverse-phase HPLC analysis showed an identical retention time for *p*NPG. *L-p*NPG: ¹H NMR (600 MHz, methanol-d₄) δ 8.20 (d, *J* = 9.3 Hz, 2H), 7.23 (d, *J* = 9.3 Hz, 2H), 5.14 (d, *J* = 7.3 Hz, 1H), 3.92 (dd, *J* = 12.4, 2.2 Hz, 1H), 3.74 (dd, *J* = 12.4, 5.7 Hz, 1H), 3.64 – 3.54 (m, 2H), 3.47 (dd, *J* = 9.3, 9.3 Hz, 1H). **MS** (ESI⁺): [M+Na]⁺ *m/z* calculated for C₁₂H₁₅NNaO₈: 324.1, found: 324.0. **RP-HPLC** (C18 HR chromolith, 100 \times 4.6 mm, 3 mL flow rate, solvent A = H₂O/TFA 99.9:0.1, solvent B = MeCN/TFA 99.9:0.1, gradient: 5 to 95 B in 5 min), *t_R* = 1.24 min.

² https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium97.pdf

β -Glucosidase was extracted from sweet almond seeds. Briefly, almond seeds (10 g) were grounded in 100 mL phosphate-buffered saline (PBS) using a kitchen hand blender. The resulting suspension was filtered through a sintered glass funnel using a sand pad to prevent clogging, centrifuged at 20,000 RPM for 30 min, and finally filtered through a 0.2 μ m membrane to obtain a clear solution, which was stored at 4 °C.

β -Glucosidase Chromogenic Assays

All experiments were performed in triplicate.

Control experiments with sweet almond β -glucosidase. Almond seed extract (30 μ L) was diluted in PBS (270 μ L), and 100 μ L of a 20 mM aqueous solution of pNPG or L-pNPG, or water (negative control) was added (final probe concentration: 5 mM). The resulting solution was then incubated at room temperature. The release of para-nitrophenolate was monitored by the experimenter.

*β -L/D-glucosidase assays on *Halobacterium salinarum*.* To 300 μ L of *H. salinarum* culture in DSMZ 97 medium, 100 μ L of a 20 mM aqueous solution of either pNPG or L-pNPG or water (final probe concentration: 5 mM) was added, and the resulting suspension was incubated at room temperature. Para-nitrophenolate release was monitored by the experimenter.

Extended Discussion

Art and Science: Towards a Synthesis

To ensure such interstellar political correctness, we tested whether our microbial astronauts, *M. sedula* and *H. salinarum*, could utilize L-sugars. We chose to design and focus on experiments with *H. salinarum* (**Figure 6**) because of its potential for glycolysis (Baati et al. 2024) and in contrast to *M. sedula*, of the cultivation modalities and the clarity of the cell-medium mixture to clearly observe a potential color activation in the previously described enzymatic hydrolysis of para-nitrophenyl- β -D-glucopyranose (pNPG), resulting in a bright yellow color.

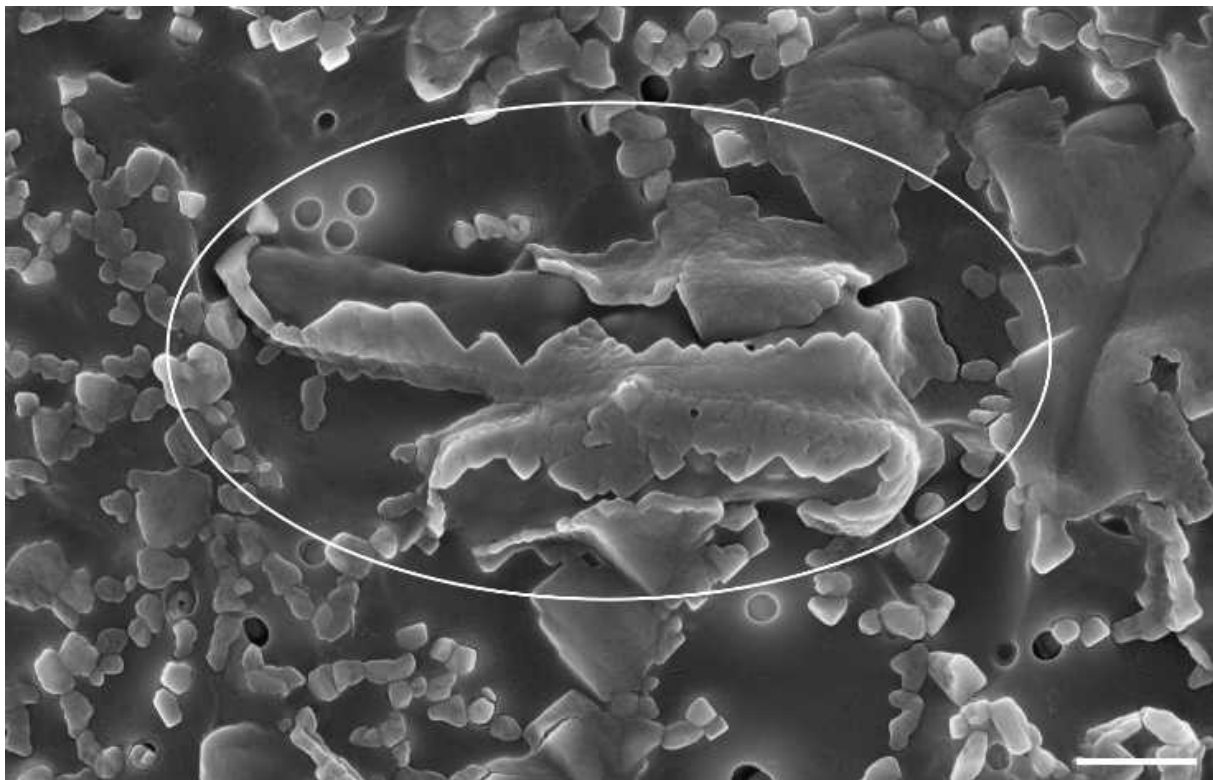


Figure 6: Scanning electron microscopy micrograph of two rod-shaped and salt-encrusted *H. salinarum* cells (white circle). Scale bar is 90 nm and is indicated in white on the bottom-right. The analysis was conducted at the Archaea Centre at the University of Regensburg (ZEISS GeminiSEM).

Glucosidase Experiment

Control experiment with sweet almond β -glucosidase. As a control experiment, we tested the activity of sweet almond β -glucosidase using both pNPG and L-pNPG probes. Incubation with pNPG rapidly produced a yellow color, reflecting the release of p-nitrophenolate upon enzymatic hydrolysis. The color intensity plateaued within 5 min, indicating complete substrate consumption. In contrast, no color change was observed with L-pNPG , even after extended incubation for up to 15 days, indicating the absence of $\beta\text{-L-glucosidase}$ activity.

Cell experiment. The viability of the microorganisms was validated before starting the experiment. During the incubation assays, no color change was observed in either pNPG- or L-pNPG -treated samples, even after 15 days, suggesting the absence of secreted β -glucosidase activity in *H. salinarum*.

Since it was also possible that other enzymes, such as nitroreductases (Akiva et al. 2017), could inactivate the chromogenic properties of pNPG and L-pNPG substrates, we added almond β -glucosidase to both samples after 15 days. This treatment led to a rapid color change to bright yellow (reaching a plateau within 5 min) in the pNPG sample, whereas no coloration was detected in the L-pNPG sample. This result confirmed that pNPG and likely also L-pNPG substrates remained intact and that pNPG could still be properly hydrolyzed by β -glucosidase.

We also considered the possibility that if present in *H. salinarum*, the β -glucosidase of our microorganism astronaut might not be secreted. To test this hypothesis, we repeated the experiment using lysed cells (cell lysis was performed by ultrasonication in an ultrasonic bath for 10 min) to release intracellular proteins into the solution. However, no reaction was observed over 15 days, and the addition of almond β -glucosidase again produced the same results as above.

In conclusion, although our assumption that “native-chirality” β -glucosidases hydrolyze only carbohydrate substrates with the natural L-configuration was consistent with the observations, no such enzymatic activity was detected in the potential microbial astronaut *H. salinarum*, with either the Earth-borne D-glucose substrate or the L-glucose mirror image. This finding suggests that *H. salinarum* could serve as a suitable Earth ambassador, safely carrying either D- or L-chirality carbohydrate analogs to probe extra-terrestrial handedness.

Although more difficult to assay, a (commercially available) sweet gift of L-glucose could be considered a “safe” present aboard the starship during the long voyage alongside the *H. salinarum* crew, as it is non-metabolizable by them, while still potentially metabolizable by mirror-image extra-terrestrial organisms.

In contrast, D-glucose is likely a less suitable option, although it has not been proven to be metabolized under our experimental conditions. It is conceivable that our microbial astronauts possess or could evolve metabolic pathways that would enable them to utilize it as an energy source.

From a practical perspective, the unprecedented β -L-glucosidase chromogenic probe prepared and rigorously characterized in this study, L-*p*PNPG, could be useful for terrestrial scientists willing to identify and measure β -L-glucosidase activities in terrestrial enzymes yet to be discovered.

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