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Microbial adaptation to ionic liquids

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Electronic Supplementary Information

Materials

Ionic liquids $C_2MIMC_2SO_4$ (>99%) and $C_2MIMC_1SO_4$ (>99%) were purchased from IoLiTec. $C_2PyC_2SO_4$ (>99%) was supplied by Merck. $P_{4441}C_1SO_4$ (>97%) was kindly donated by CYTEC industries. All of them were subjected to vacuum ($P = 2 \cdot 10^{-1}$ Pa) and moderate temperature ($T = 343.15$ K) for several days to remove possible traces of solvents and moisture, always prior to their use. The ionic liquids were kept in bottles under inert atmosphere until use.

Microorganisms and culture medium

Pseudomonas stutzeri, *Staphylococcus warneri* and consortium C26b were grown in a mineral medium composed of: $Na_2HPO_42H_2O$ 8.5 g/L, KH_2PO_4 3.0 g/L, NaCl 0.5 g/L, NH_4Cl 1.0 g/L, $MgSO4 \cdot 7H_2O$ 0.5 g/L, $CaCl_2$ $14.7 \cdot 10^{-3}$ g/L, $CuSO_4$ 0.4 mg/L, KI 1.0 mg/L, $MnSO_4 \cdot H_2O$ 4.0 mg/L, $ZnSO_4 \cdot 7H_2O$ 4.0 mg/L, H_3BO_3 5.0 mg/L, $FeCl_3 \cdot 6H_2O$ 2.0 mg/L. 15 g/L of glucose were also included in the culture medium. [1,2]

Trametes versicolor and *Shewanella oneidensis* were grown in a medium containing glucose (15 g/L), yeast extract (15 g/L), NH_4Cl (0.75 g/L), KH_2PO_4 (2 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), $CaCl_2 \cdot 2H_2O$ (0.1 g/L) and KCl (0.5 g/L).[3]

Halobacterium salinarum and *Anoxybacillus flavithermus* were cultured in Luria-Bertani medium, composed of 10 g/L trypticase, 5 g/L yeast extract and 10 g/L NaCl. The latter was isolated in a Galician hot spring, as previously reported.[4,5]

Phanerochaete chrysosporium BKM-F-1767 (ATCC 24725) was grown in Kirk medium, as reported elsewhere.[6]

Thermus thermophilus HB27 was kindly provided by Dr. J. Berenguer (Universidad Autonoma, Madrid, Spain). This microorganism was grown in a

liquid medium containing (g/L, in distilled water) 8 trypticase, 4 yeast extract and 3 NaCl.[7]

The microorganism inocula were obtained by cultivating the microorganisms in 250 mL-flasks capped with cellulose stoppers, containing the corresponding culture media, and after the stationary phase was reached, the biomass was separated by centrifugation at 5.000xg for 10 min, at 4ºC. The humidity was removed by vacuum drying and the dried cells (pellets) were stored at -20ºC in Eppendorf tubes. The same amount of cells was used for subsequent cultures, since each pellet (with a given cell concentration determined after a calibration absorbance at 600 nm vs. dried cell weight) was resuspended in the corresponding IL-containing culture medium, and it was used to inoculate 100 mL of medium (to yield an initial biomass concentration of 3%).

Effect of ionic liquids on microorganisms

Two different media were used for investigating the effect of the three selected ionic liquid families: a mineral (MM) and a rich medium (RM) with compositions detailed by Moscoso et al (2012)[1] and Deive et al (2010),[5] respectively.

The pH of each medium was adjusted according to the previously reported, namely: *P. chrysosporium* pH 4.5, *T. versicolor* and *S. oneidensis* pH 7, consortium C26b, *St. warneri*, *A. flavithermus*, *A. salinarum* and *T. thermophilus* HB27 pH 7.5, and *P. stutzeri* pH 7.2. All the liquid media were sterilized by autoclaving at 121ºC for 20 min. They were then inoculated with the corresponding microorganisms at 37ºC for the mesophiles, 60ºC for *A. flavithermus* and 70ºC for *T. thermophilus* HB27. The cultures were carried out in 96 well plates containing different concentrations of ionic liquids (between 0.005M to 1.5 M). MLC was ascertaining by inoculating plates without the ionic liquid pressure.

Microscopy analysis

Scanning electron microscopy (SEM) images were taken in a FEI-Quanta 200 environmental scanning electron micro-scope using an accelerating voltage of 15 kV (Electron Microscopy Service, C.A.C.T.I., University of Vigo).

Microbial acclimation

The stirred tank bioreactor (Biostat B, Sartorius, Germany) consisted of a 3L-jacketed glass column filled with 2 L of mineral medium described above containing 200 μM of $C_2MIMC_2SO_4$. The temperature was maintained at 37 ºC by circulation of thermostatted water, and the pH was adjusted to 8. The bioreactor was inoculated with actively growing cells of *P. stutzeri* from 24 h flask cultures (3% v/v). Humidified air was supplied continuously at 0.17 vvm and the agitation was set at 100 rpm. Bioreactor was operated for two months.

Biopolymer production

The adapted *P. stutzeri* was cultured in 250 mL Erlenmeyer flasks containing 50 mL of mineral medium. After 48 h, maximum biopolymer concentration was detected. Therefore, the culture medium was centrifuged at 14000 rpm and 4ºC to separate the cells. The supernatant was reserved for biopolymer recovery. Afterwards, a mixture containing the supernatant and ethanol in a 1:2 ratio was kept at 4ºC for 2 hours and then centrifuged at 14000 rpm and 4ºC.

Biopolymer characterization

The biopolymer composition was ascertained after a preliminary acid hydrolysis step, followed by the injection in an HPLC (Agilent 1100) equipped with a RI detector. Composition determination was carried out by direct comparison with standards such as glucose, fructose, sucrose, maltose and rhamnose.

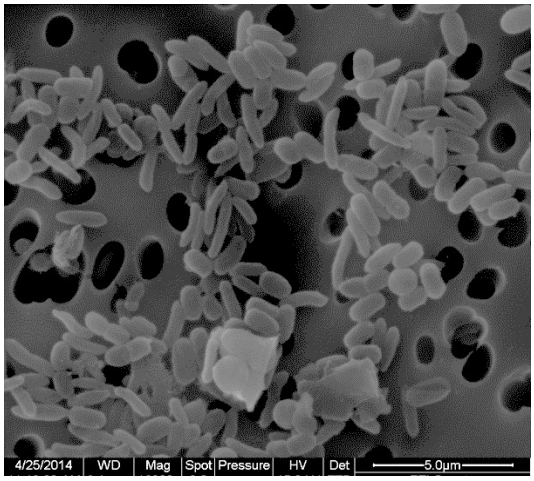

Fig. S1 SEM image of *P. stutzeri* after two months in a stirred tank bioreactor without the presence of the ionic liquid

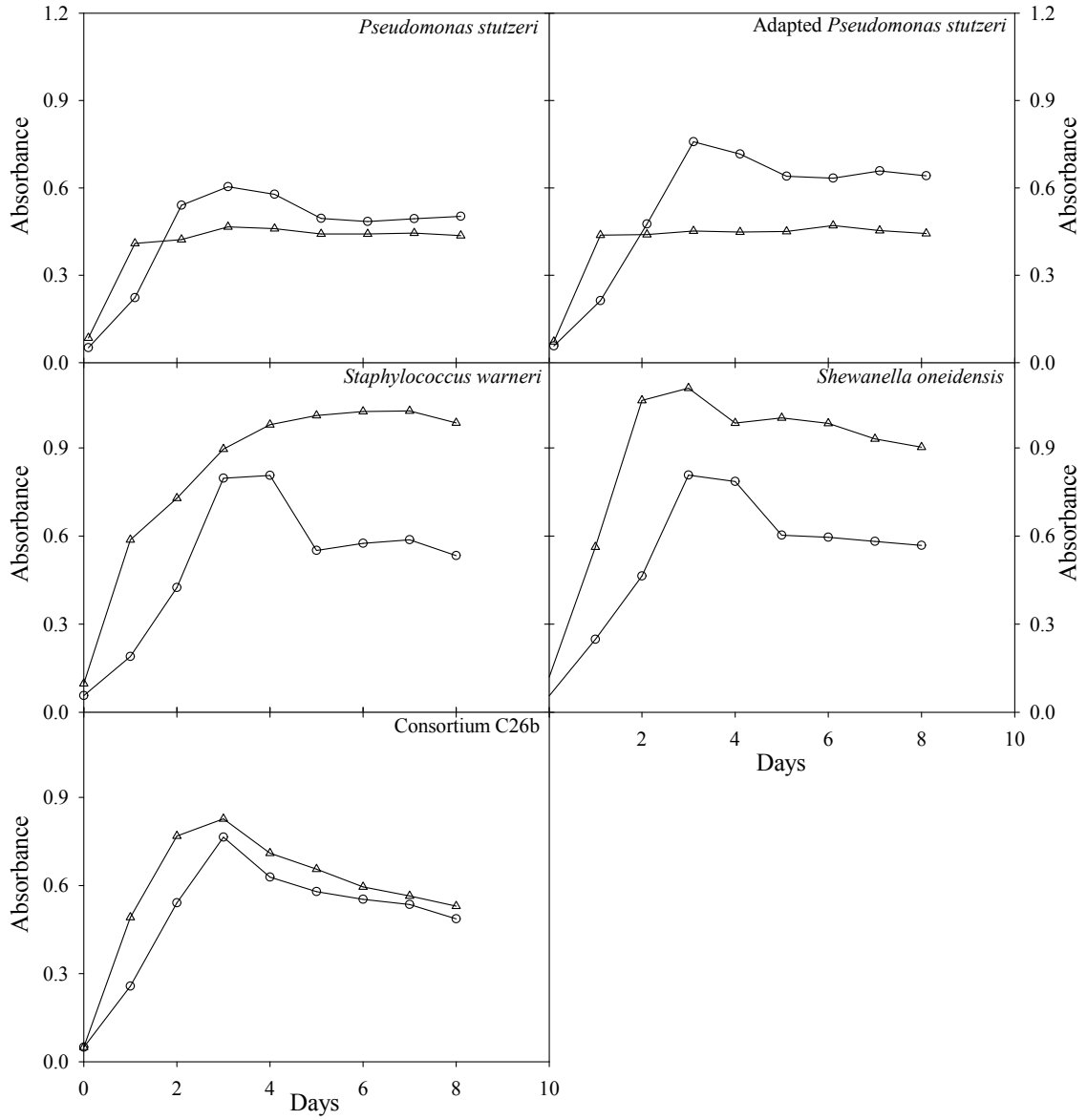

Fig. S2 Microbial growth in the absence of ionic liquids in rich (❒) and mineral (ᔓ) medium of *Pseudomonas stutzeri*, adapted *Pseudomonas stutzeri*, *Staphylococus warneri*, *Shewanella oneidensis* and Consortium C26b.

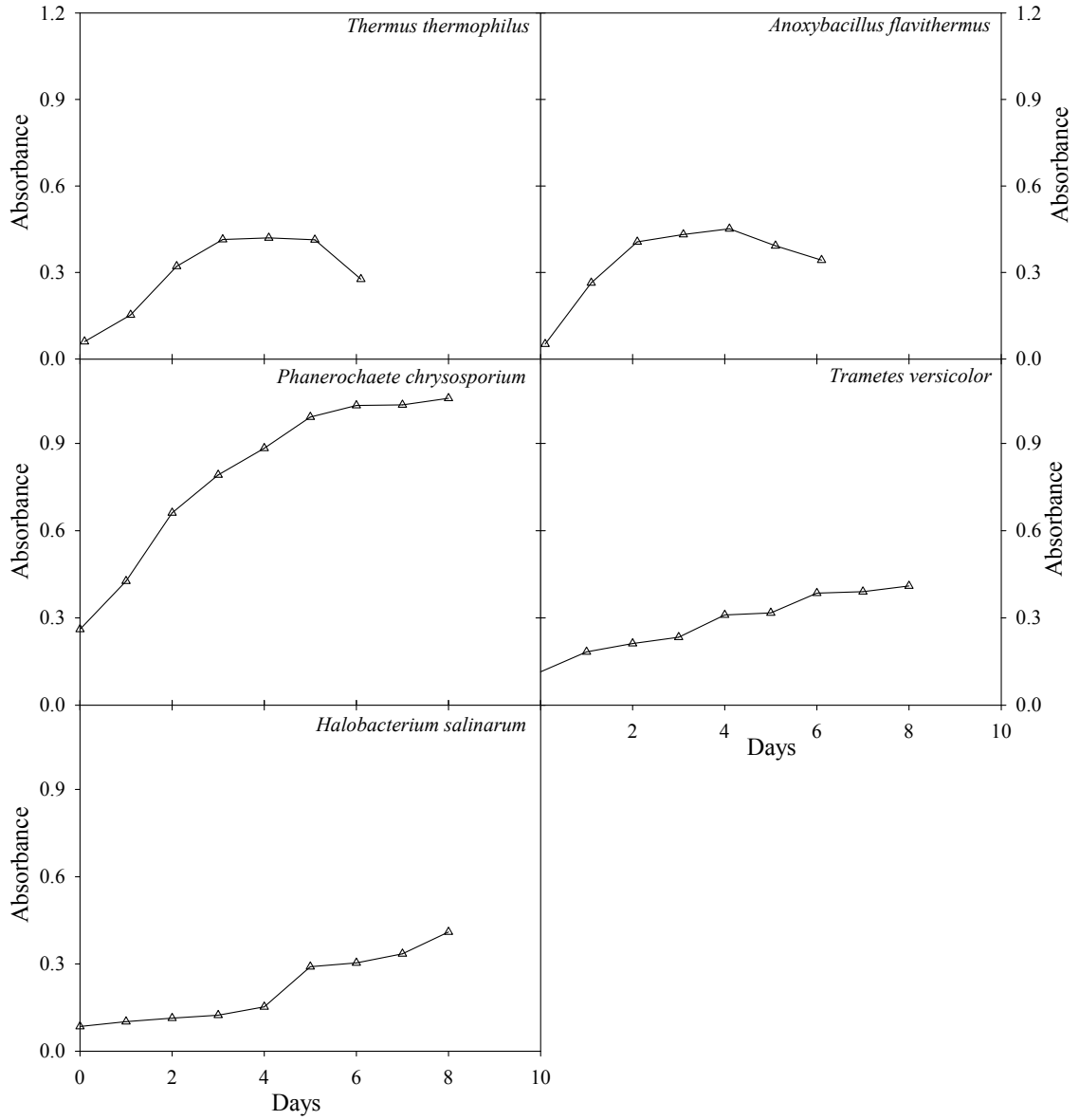

Fig. S3 Microbial growth in the absence of ionic liquids in rich medium of *Thermus thermophilus*, *Anoxybacillus flavithermus*, *Trametes versicolor*, *Phanerochaete chrysosporium* and *Halobacterium salinarum*

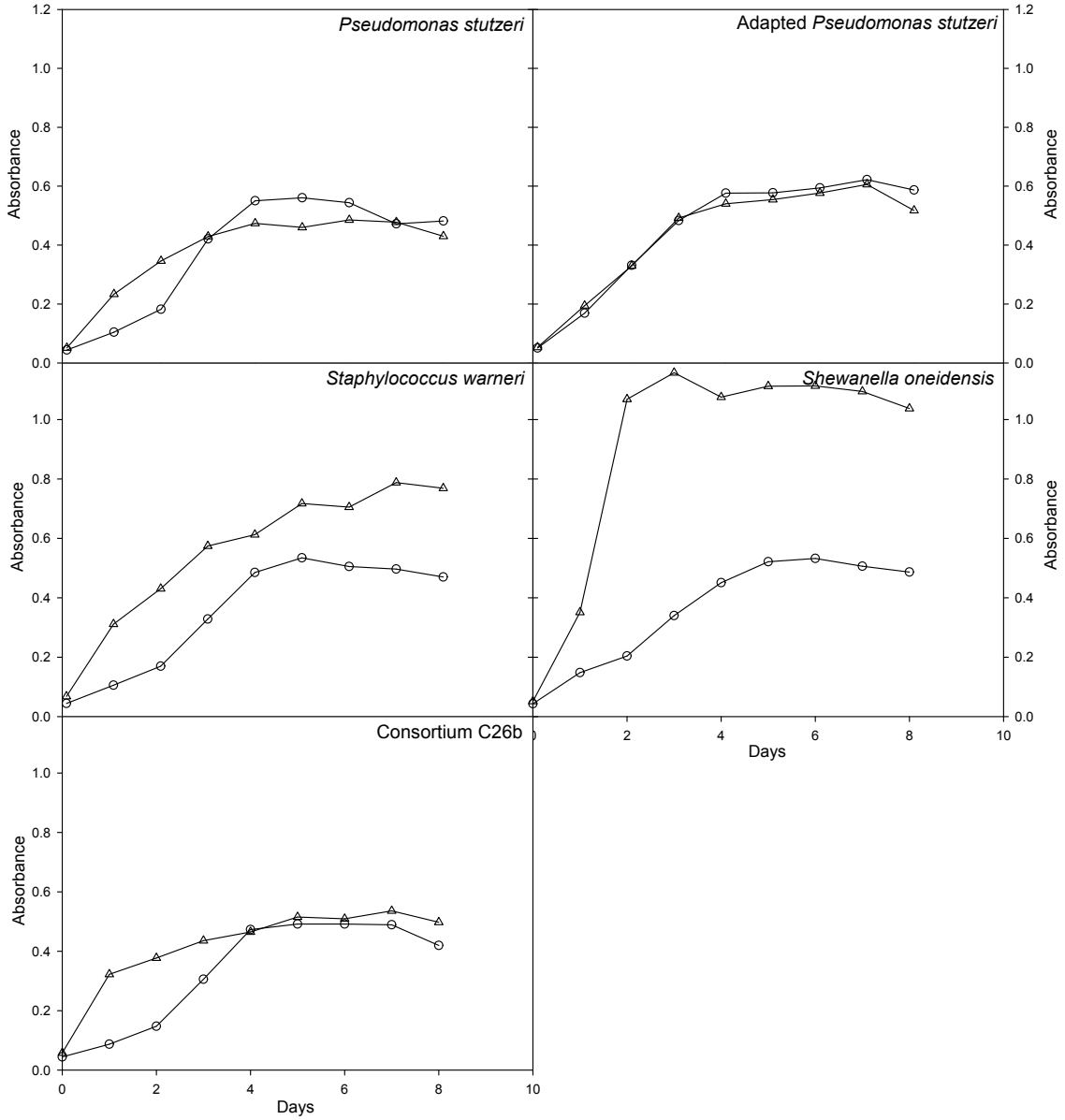

Fig. S4 Microbial growth at MIC concentration of $C_2MIMC_1SO_4$ in rich (❒) and mineral (ᘓ) medium of *Pseudomonas stutzeri*, adapted *Pseudomonas stutzeri*, *Staphylococus warneri*, *Shewanella oneidensis* and Consortium C26b.

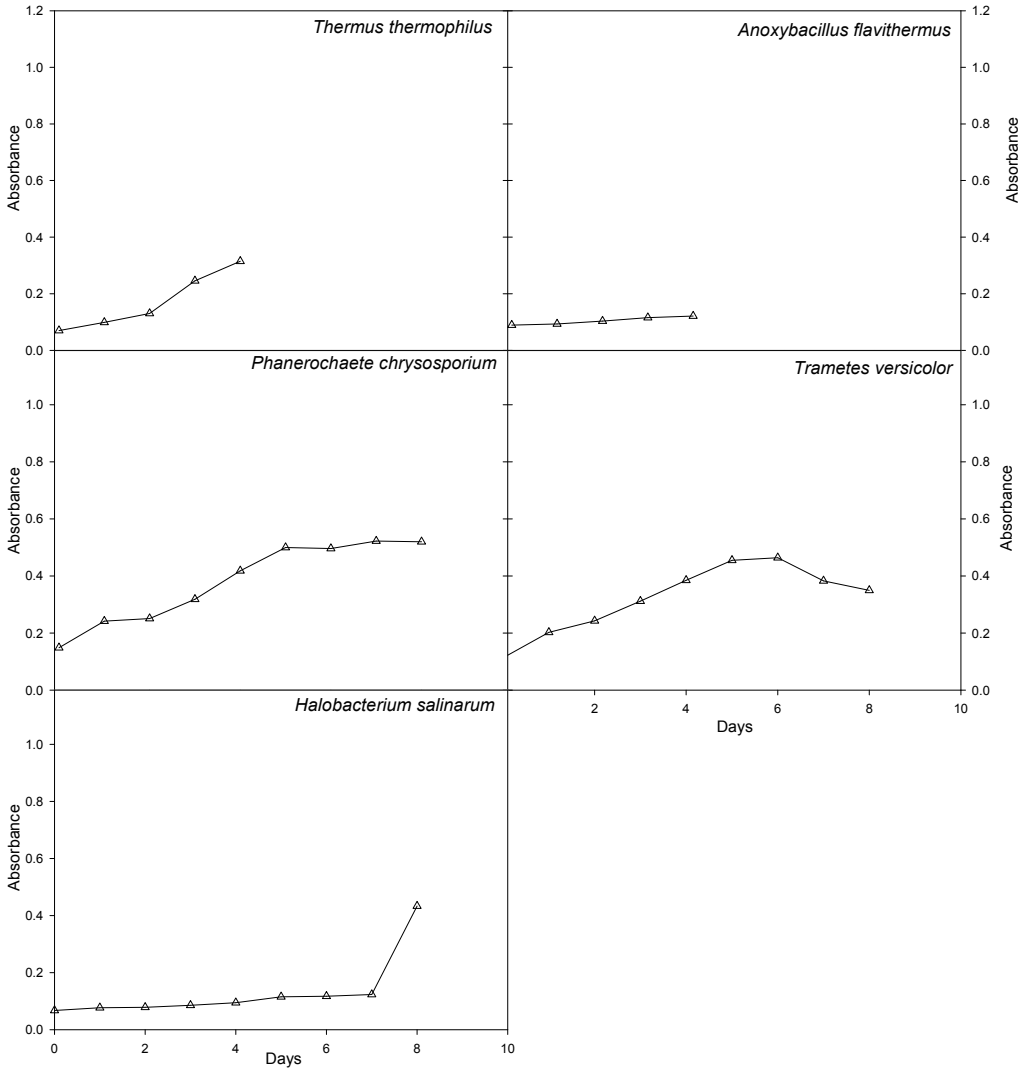

Fig. S5 Microbial growth at MIC concentration of $C_2MIMC_1SO_4$ in rich medium of *Thermus thermophilus*, *Anoxybacillus flavithermus*, *Trametes versicolor*, *Phanerochaete chrysosporium* and *Halobacterium salinarum*

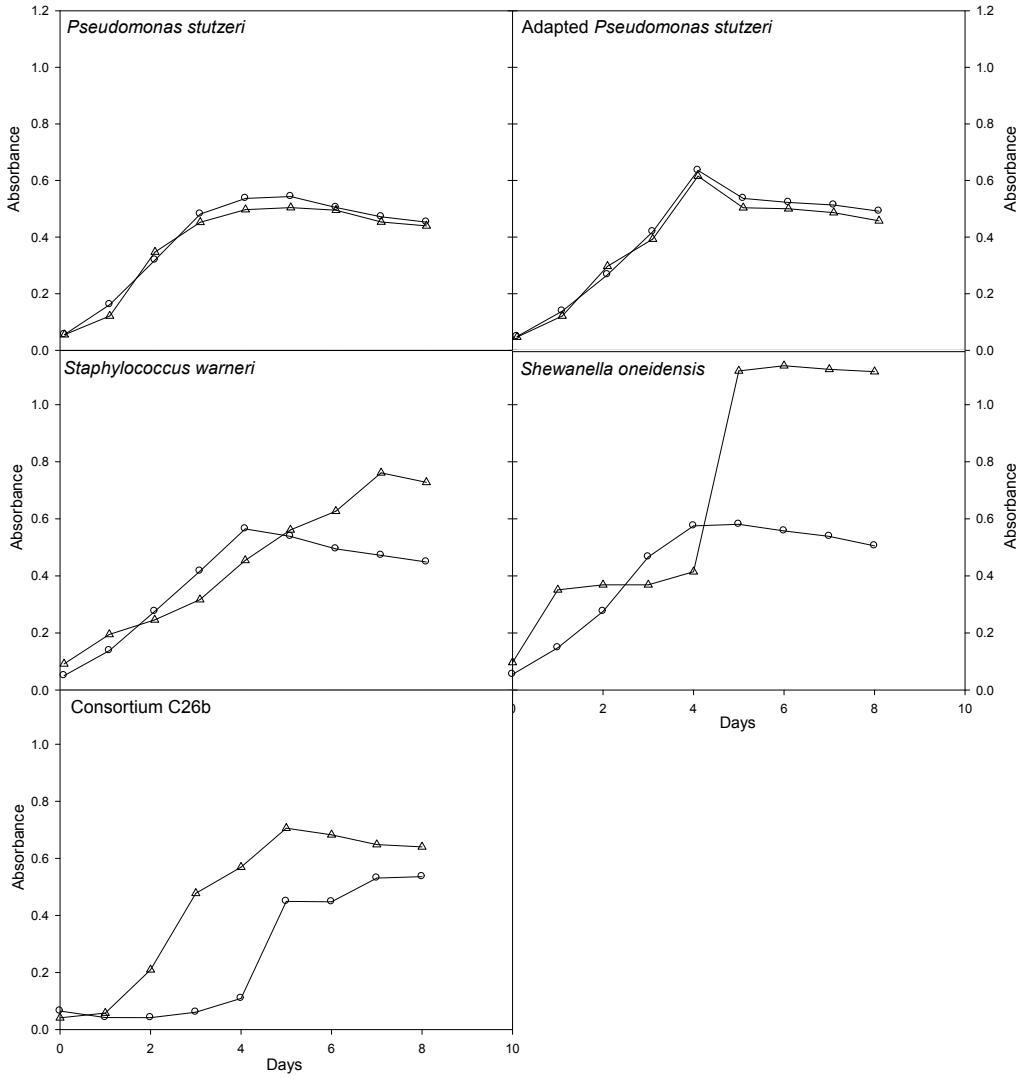

Fig. S6 Microbial growth at MIC concentration of $C_2MIMC_2SO_4$ in rich (❒) and mineral (ᔓ) medium of *Pseudomonas stutzeri*, adapted *Pseudomonas stutzeri*, *Staphylococus warneri*, *Shewanella oneidensis* and Consortium C26b

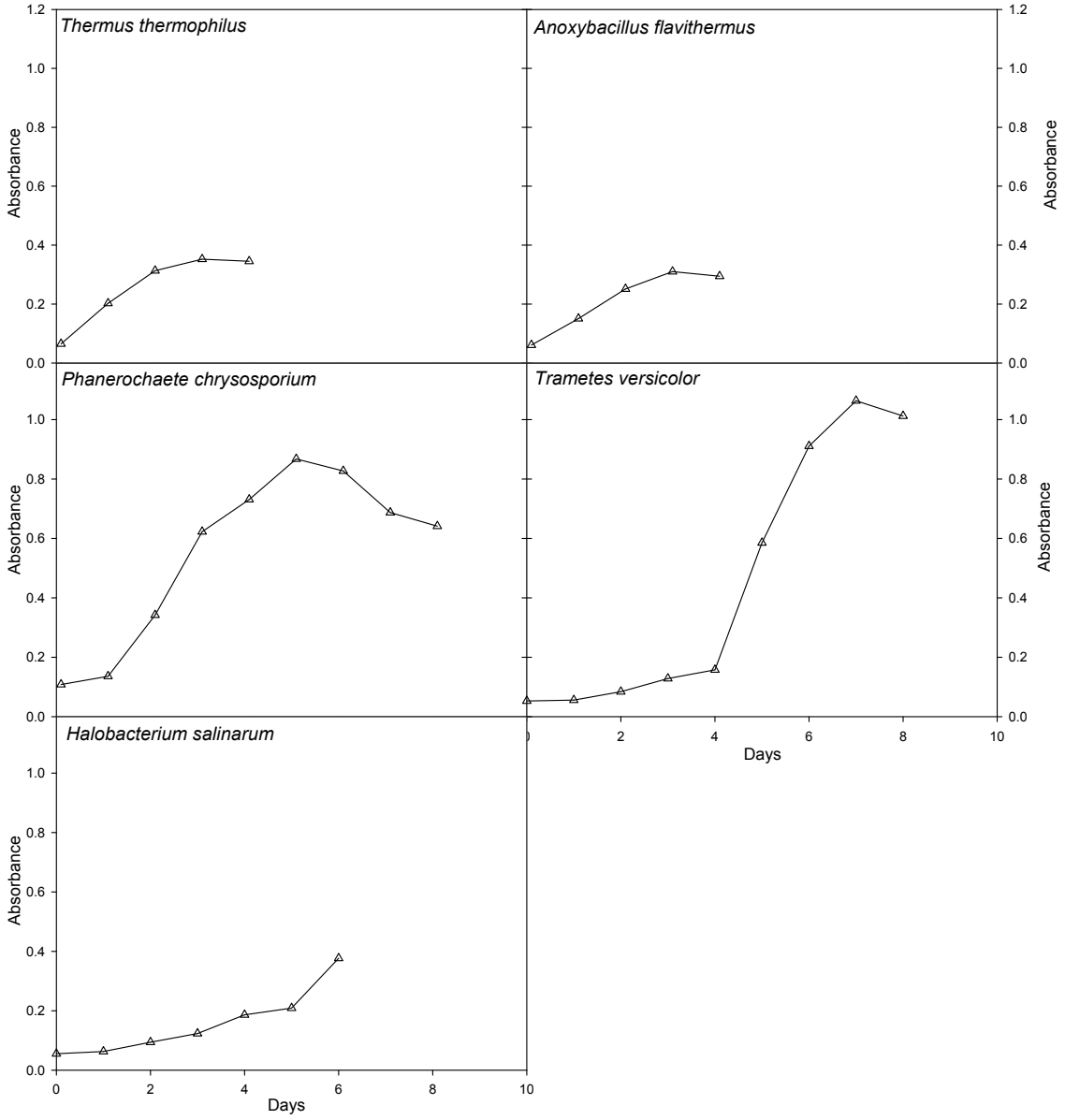

Fig. S7 Microbial growth at MIC concentration of $C_2MIMC_2SO_4$ in rich medium of *Thermus thermophilus*, *Anoxybacillus flavithermus*, *Trametes versicolor*, *Phanerochaete chrysosporium* and *Halobacterium salinarum*

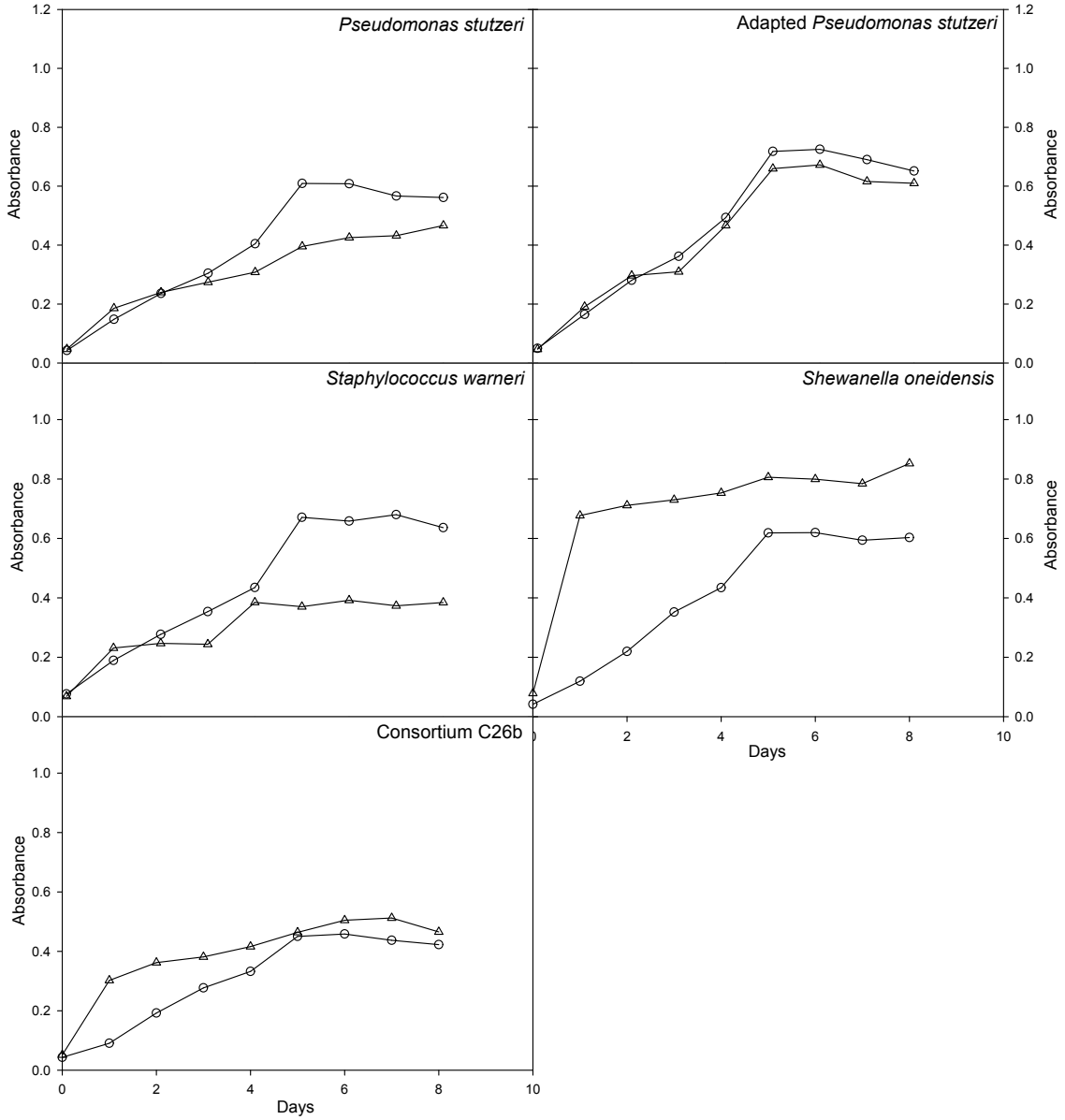

Fig. S8 Microbial growth at MIC concentration of $C_2PyC_2SO_4$ in rich (❒) and mineral (ᘓ) medium of *Pseudomonas stutzeri*, adapted *Pseudomonas stutzeri*, *Staphylococus warneri*, *Shewanella oneidensis* and Consortium C26b

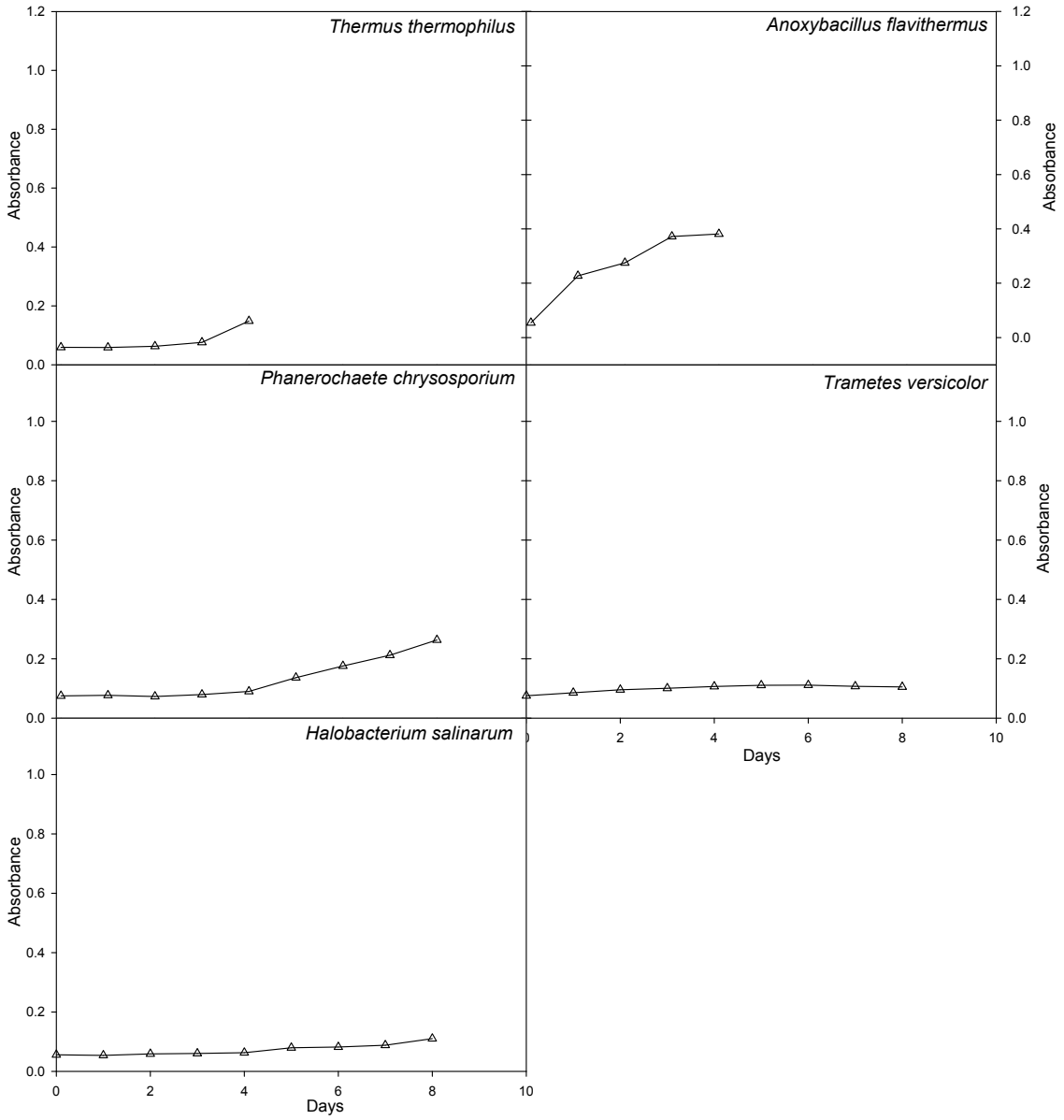

Fig. S9 Microbial growth at MIC concentration of $C_2PyC_2SO_4$ in rich medium of *Thermus thermophilus*, *Anoxybacillus flavithermus*, *Trametes versicolor*, *Phanerochaete chrysosporium* and *Halobacterium salinarum*

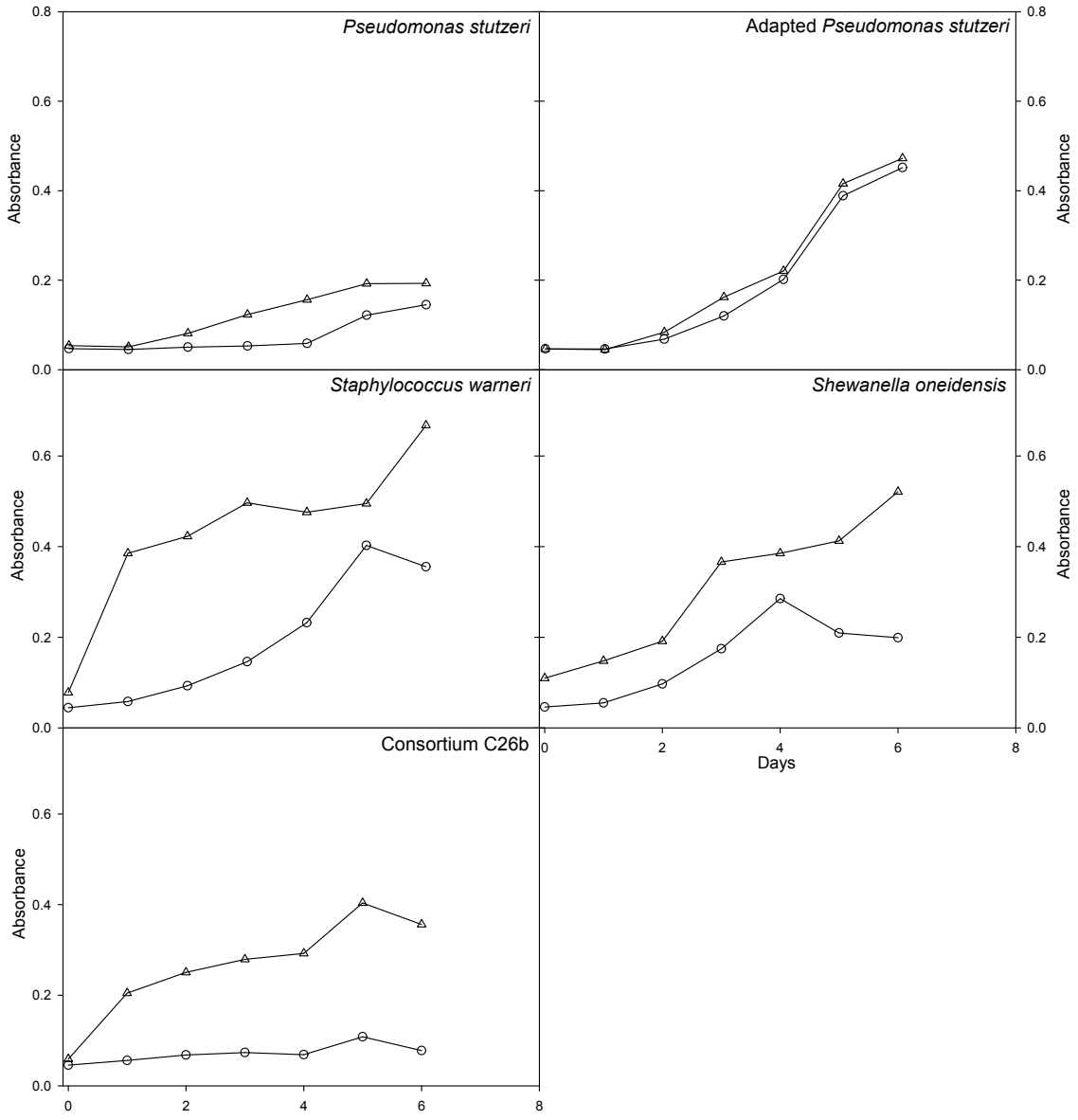

Fig. S10 Microbial growth at MIC concentration of $P_{4441}C_1SO_4$ in rich (❒) and mineral (ᘓ) medium of *Pseudomonas stutzeri*, adapted *Pseudomonas stutzeri*, *Staphylococus warneri*, *Shewanella oneidensis* and Consortium C26b

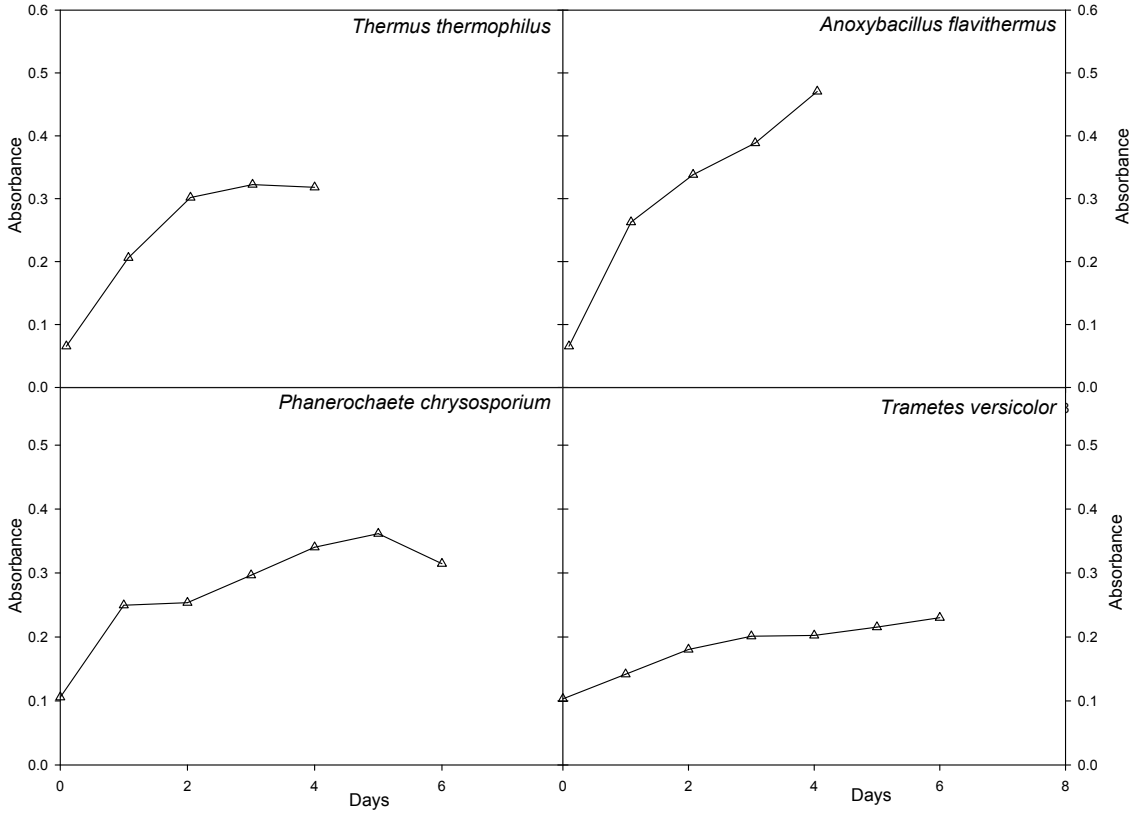

Fig. S11 Microbial growth at MIC concentration of $P_{4441}C_1SO_4$ in rich medium of *Thermus thermophilus*, *Anoxybacillus flavithermus*, *Trametes versicolor*, *Phanerochaete chrysosporium* and *Halobacterium salinarum*

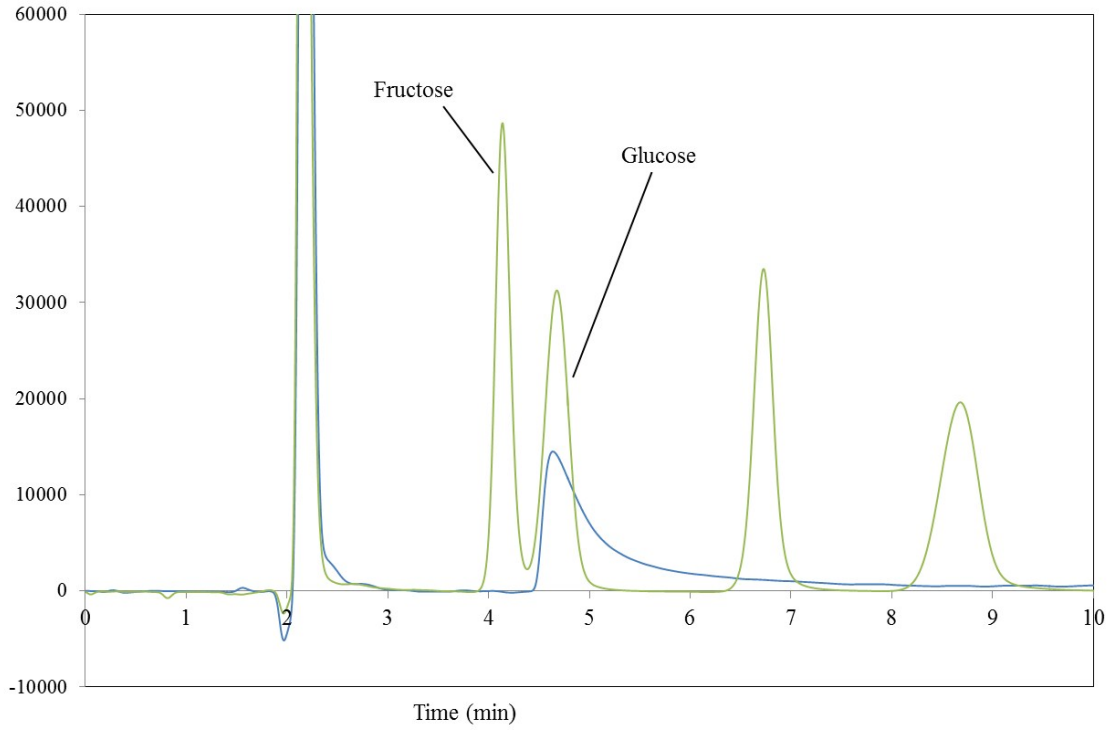

Fig. S12 HPLC chromatogram of the hydrolysed biopolymer (blue) and standard of conventional oligosaccharides (green).

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