Supplementary Information for:

A cryogel-based bioreactor for water treatment applications

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Summary: this supplementary information contains 9 figures and 2 tables in 19 pages.

2. Materials and Methods.

Two known strains of phenol-degrading bacteria *Pseudomonas mendocina* (*P. mendocina or Pse)* (NCIMB 13264) and *Rhodococcus koreensis* (*R. koreensis or Rho)* (NCIMB 13709) were purchased from the National Collection of Industrial, Food and Marine Bacteria, UK. *Acinetobacter radioresistens* (*A. radioresistens or Acn*) was obtained from the culture collection PABS, UoB (Acinetobacter 16S rRNA analysis is given in supplementary information). Tryptone soya broth (TSB), tryptone soya agar (TSA) and phosphate buffered saline (PBS) were obtained from Oxoid Ltd, UK. Live/Dead Bac Light kit (L7007) sodium chloride, phenol, ammonium chloride, Gellan Gum (Gel)(technical grade) were purchased from Fisher Scientific Co, UK. Fluorescein isothiocyanate (FITC) and rhodamine B were purchased from Sigma Aldrich, UK. 4-aminoantipyrine, glutaraldehyde (GA) solution 50 w/v %, polyvinyl alcohol (PVA) (deacetylation degree 86.7-88.7%, Mw 67,000 Da), polyethyleneimine, linear (Mw 423 Da), chitosan (CHI) (medium viscosity), Pur-A-Lyzer TM Mega 1000 Dialysis Kit (1000 Da) and (14 kDa) pore size were purchased from Sigma-Aldrich. Potassium ferricyanide and ammonium hydroxide (28-30 wt/v % solution of NH_4OH in water) Gellan Gum were obtained from Acros Organics, UK.

2.1 *Acinetobacter* 16S rRNA analysis

The presumptive *Acinetobacter* isolates were characterised biochemically by Gram staining, growth on differential and enriched media, the oxidase test, the catalase test, the motility test,(MacFaddin et al., 2000 and 2000) the Phenylalanine Deaminase test,(MacFaddin et al., 2000) and with API 20E and API 20NE kits (BioMerieux, France). Isolates identified as presumptive *Acinetobacter* species were further identified using 16S rRNA analysis.

Molecular identification was performed via the polymerase chain reaction (PCR), using the 16S rRNA universal primers (27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-ACCTTGTTACGACTT-3') for DNA amplification.(Weisburg et al., 1991; Jiang et.al 2013) DNeasy TM Blood & Tissue kit (Qiagen, UK) was used to extract and purify DNA following the manufacturer's instructions. The extracted DNA was diluted with biologically sterilised water at a ratio of 1:10 and mixed using a Mega Mix- Royal solution kit (Microzone, UK), containing Taq polymerase, 2x enhancing buffer (6 mM MgCl2) with 400 µM dNTPs and blue MiZN loading dye and stabiliser, prior to PCR. The stock DNA extractions were stored at -20oC for further use.

A 12.5µl volume of Mega Mix- Royal solution was added to an equal volume of DNA, primer and water to give 25 µl total volume to activate the tag polymerase. Following initial denaturation at 95ºC for 5 minutes the PCR thermocycler (G-Storm, LabTech International, UK) was run for 30 cycles of 95ºC for 1 minute, 50ºC for 45 seconds, with extension at 72ºC for 1.5 minutes and a final extension of 72ºC for 10 minutes.

Separation and visualisation of the amplified DNA fragments was achieved through agarose gel electrophoresis of 10 µl of amplification products with an agarose concentration of 1.5% agarose. A size ladder up to 4500pb was used with distilled water as a control, with electrophoresed Tris-Acetic-EDTA (TAE) buffer for three hours at 100 volts on a HU15 horizontal gel units (LabTech International, UK).

After electrophoresis the gels were stained using ethidium bromide at a concentration 0.5% for 15 minutes then, after washing in water, imaged and documented using the SynGene programme and SynGene INGENIUS UV imager(Fig. S1). Identification of DNA sequences was performed on 25 µl aliquots of amplification products at a concentration of 20ng/µl by the Eurofins Genomics DNA sequencing service.

The API20NE biochemical identification was confirmed by comparison of the identity code (0000032) with the API database online; this resulted in a 96.7% similarity to *Acinetobacter radioresistens*.

The result of 16s rRNA sequences of *Acinetobacter radioresistens* were compared with those in the Genebank database through BLAST analysis, using the Greengen's website (http://greengenes.lbl.gov/cgi-bin/JD_Tutorial/nph-16S.cgi). From this the identity of the presumptive phenol-degrading *Acinetobacter* isolates were identified as *Acinetobacter radioresistens* strains SK82 at a probability of 99.80% and 100% for the forward and reverse primers.

2.2 Estimation of toxicity of cross-linking polymers.

Cultured media (80 mL) was centrifuged at 10 000 rpm for 10 minutes at 4ºC. The obtained pellet was re-suspended in PBS buffer. The 0.5 mL suspension of bacteria in PBS buffer was used as a positive control. 0.5 mL of the suspension was mixed with PEI-al, PVA-al, and PVA polymers, and a combination PEI-al & PVA-al. GA (0.25 and 0.5 v/v %) was used as a negative control. Part of the samples were kept at 4 ºC for 24h, while the other part of the same composition solution was frozen at -12 ºC and kept frozen for 24h. Then, the frozen samples were defrosted at room temperature and the number of live bacteria estimated using MTT assay.

The viability of cross-linked bacteria within the cryogel structure was evaluated using the LIVE/DEAD® bacterial viability kit and confocal laser scanning microscopy (CLSM, Leica TCS SP5). Hydrogel-Rho-Gellan0.5% Hydrogel-Rho-Gellan(1.0%) and Cryo-Rho-

Gellan(0.5%) PVAal (0.3%) cryogels were cut into slices with thickness of 1 mm. The samples were rinsed twice with 0.9% NaCl buffer and stained with SYTO 9 stain (wavelength 480/500 nm) and propidium iodide (wavelength 490/635 nm) for 15 minutes at room temperature under dark conditions according to the protocol of the LIVE/DEAD ® Bacterial viability kit.

2.3 Monitoring of phenol derivative concentration

For assessment of phenol, m-cresol, 2-CP and 4-CP concentration, samples of 100 µL volume were transferred into an eppendorf tube (1.5 mL) and diluted with water (0.8 mL). Fifty µL of ammonium buffer was added to adjust pH to ten. 4-aminoantipyrine (25 µL, 20 mg/mL) solution followed by 25 µL potassium ferricyanide (0.242 mol/L) was added. The mixtures were vortexed and centrifuged at 13 000 rpm and absorbance at 510 nm was measured. The final concentrations of phenol derivatives and their possible by-products in water after degradation by the 3D, cryostructured, macroporous bioreactors (henceforth termed cryobacteria reactors, or CBRs) were estimated using an Agilent 1100 HPLC. All samples were diluted 1:1 with HPLC grade methanol and frozen at -20 ºC for 2-3h to precipitate unwanted compounds. Samples were centrifuged at 10 000 rpm for five minutes and filtered through 0.22-µm filter membranes before HPLC analysis using a UV detector at 280 nm. A reverse phase column C18 was used, and the flow rate of the mobile phase was 1 mL/min.

HPLC method. Mobile phase Acetic acid 1 % v/v in water (C) and methanol (D) was used. Column and sample temperatures were 45 ºC and 25 ºC, respectively; C) gradient start at 75.0, End at 44.0 %; D) gradient start at 25.0, end at 56.0 % for 9.5 min, and at composition 44 % - 56 % C-D for 5 min; C) gradient start at 44.0, end at 0 % with duration of 1.0 min and D) gradient start at 56.0 %, end at 100.0 %, for 1.0 min. 5 minutes at 100 % of CH_3OH & 1 % HAc. C) gradient start at 0, end at 75.0 % for 1.0 min; D) gradient start at 100.0, end at 25.0 % with duration of 1.0 min. Retention time (min) used: phenol (5.58-6.6), p-cresol (8.88), m-cresol (8.99), p-hydroxybenzoic acid (3.4), hydroquinone (1.97), protocatechuate (2.396), 2-chlorophenol (9.44), 4-chlorophenol(10.75).

3. Results

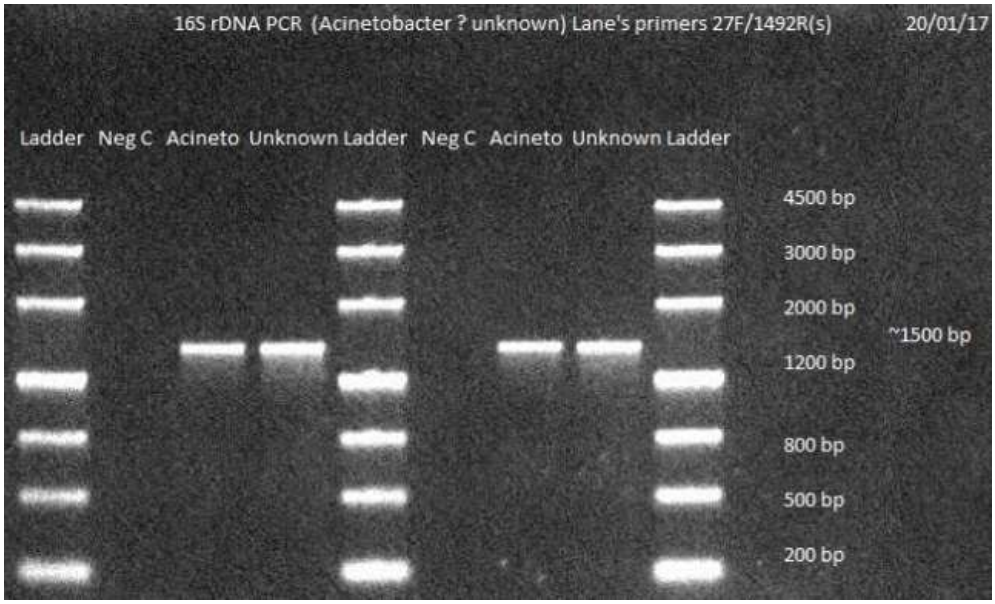
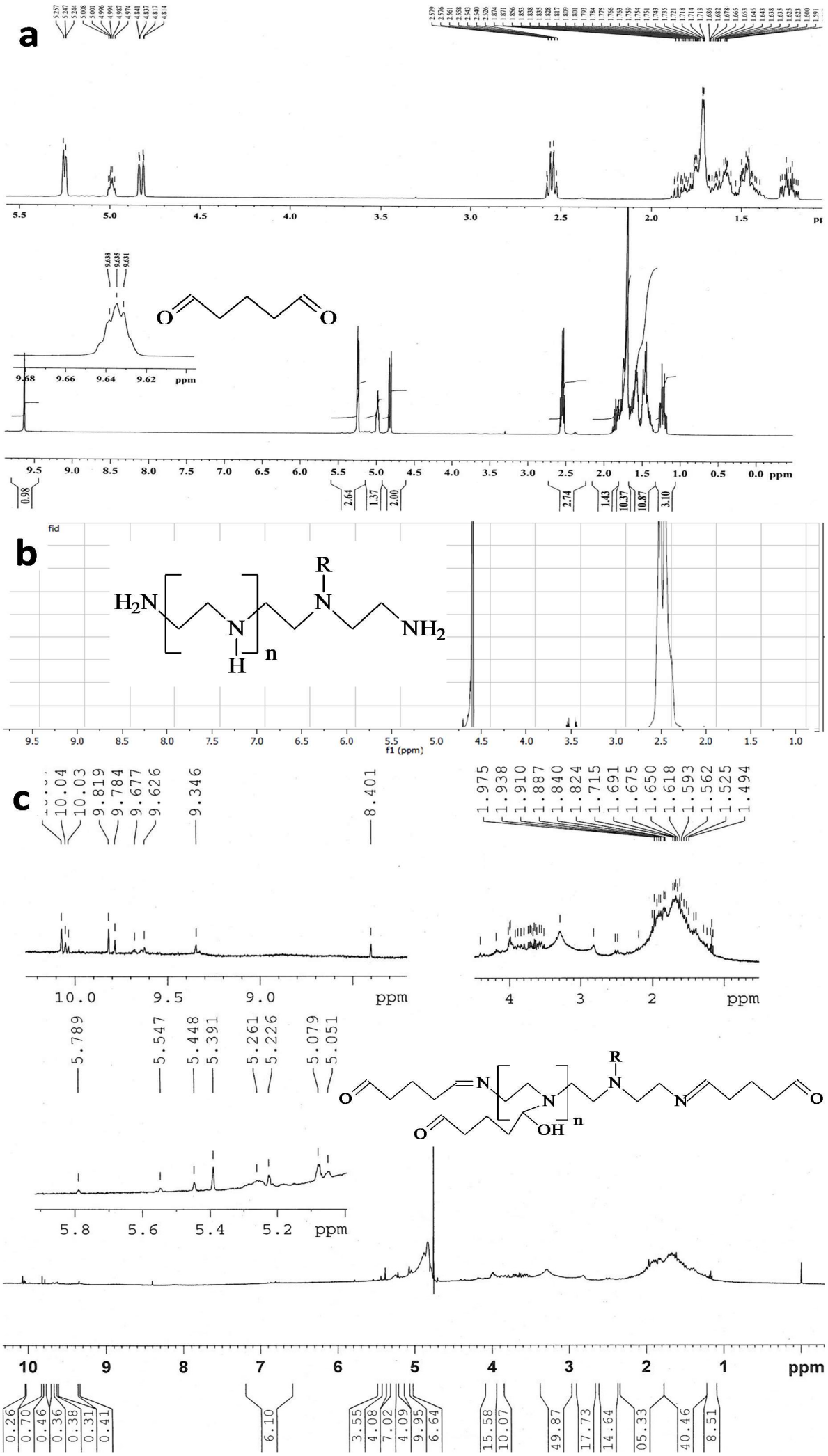


Fig. S1. Electrophoresis of 16S rRNA *Acinetobacter* unknown(Gramm negative, coccus/coccobacillus, oxidise negative, catalase positive, non-fermentative) and comparison with *Acinetobacter radioresistence*.


a
b
c
fid
f1 (ppm)
ppm

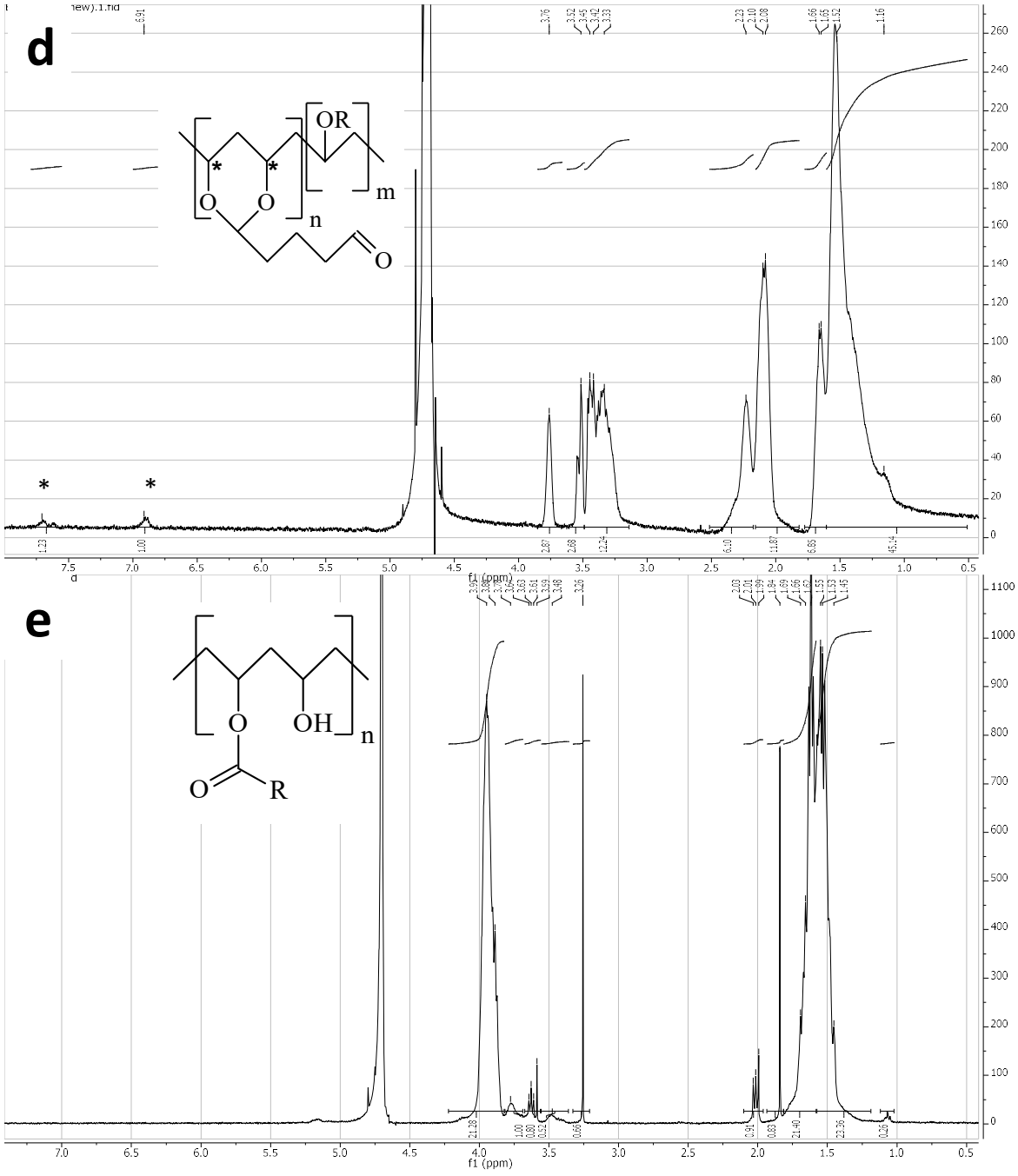


Fig. S2. H-NMR-spectra recorded in D_2O of: a) glutaraldehyde, b) PEI, c) PEI-al, d) PVA-al, e) PVA.

Characterisation of new polymers and morphology of materials

A group of multiplets in the range of 3.55-3.70 ppm in the spectra (Fig. S1a) corresponds to backbone protons of PEI, which shifted after the modification of the polymer. The condensation reaction of the primary and secondary amino groups of PEI with GA leads to the disappearance of amine functionality at 2.6 ppm (Fig. S1b). The backbone protons of GA

119 moiety appeared at 1.25-1.75 ppm and in the field of 5ppm attributed to hemiacetal form of
120 GA (Fig. S1d)(Wang et al. 2014). Aldehyde groups were observed at 9.6 ppm (Fig. S1a). The
121 proton of Schiff's base in the ^{1}H-NMR spectrum of PEI-al appeared at 8.36 ppm (Ederer et al.,
122 2007; Boghaei et al., 2008; Makhubela et al., 2011). PVA-al has two additional signals at 7.7
123 and 6.3 ppm which are attributed to presence of protons of the cyclic acetal form and multiplets
124 at 2.23 ppm related to glutaraldehyde moieties(CH_2(4H)) and the singlets at 2.1ppm attributed
125 to acethyl groups(CH_3) of PVA (Fig. S1c). Taking into account the relation of integrals of
126 peaks for backbone -(**CH_2**-CH(R))n- of PVA at 1.6ppm and the characteristic signal of
127 glutaraldehyde (CH_2(4H)) one can conclude that the ration between structural unit of PVA to
128 GA was 14.8 : 1(Fig. S1c). FTIR spectra of PEI-al and PVA-al showed carbonyl groups at
129 1714 cm^{-1} and 1717 cm^{-1}, respectively, which indicated the presence of free aldehyde groups
130 in the polymer structure.
131

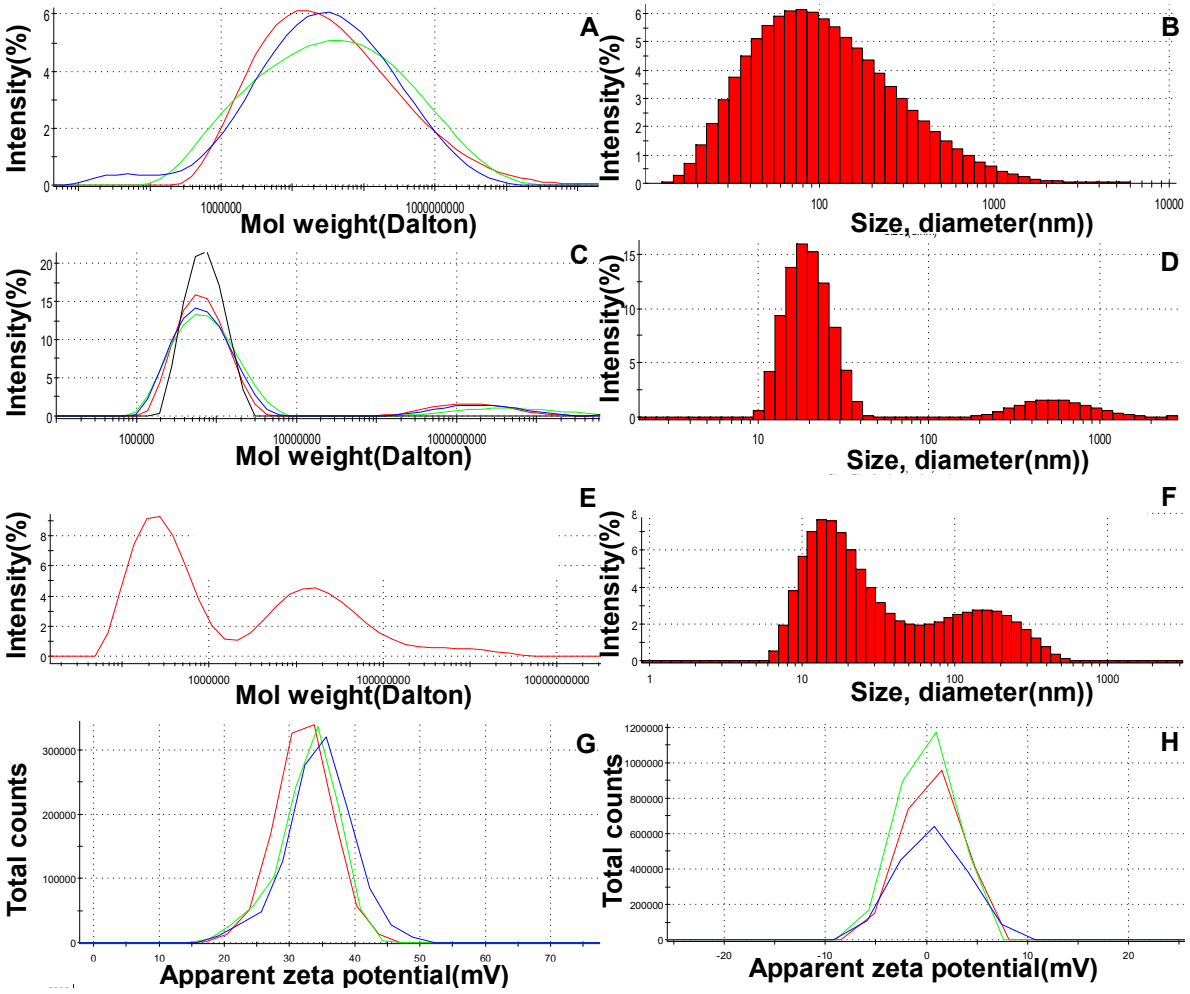


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Fig. S3. Hydrodynamic weight distribution of polymers: a & b) PEI-al; c & d) PVA; e & f) PVA-al. Zeta potential of polymers in water: g) PEI-al; h) PVA-al.

The concentration of aldehyde groups in PVA-al was estimated using a known reaction with hydroxylamine and subsequent titration of released hydrochloric acid(Zhao et al., 1991). Thus, the 2% PVA-al solution contained 6.25 mM/L of aldehyde groups, while the 1% PEI-al solution contained approximately 57 mM/L of aldehyde groups, calculated via measuring the 2.4-dinitrophenylhydrazine assay, which was used previously for estimation of amount of aldehyde groups in oxidised dextran(Berillo et al. 2012). PVA-al and PEI-al contained 0.3125 $mM/g_{dry\ polymer}$ and 5.7 $mM/g_{dry\ polymer}$ of aldehyde groups, respectively.

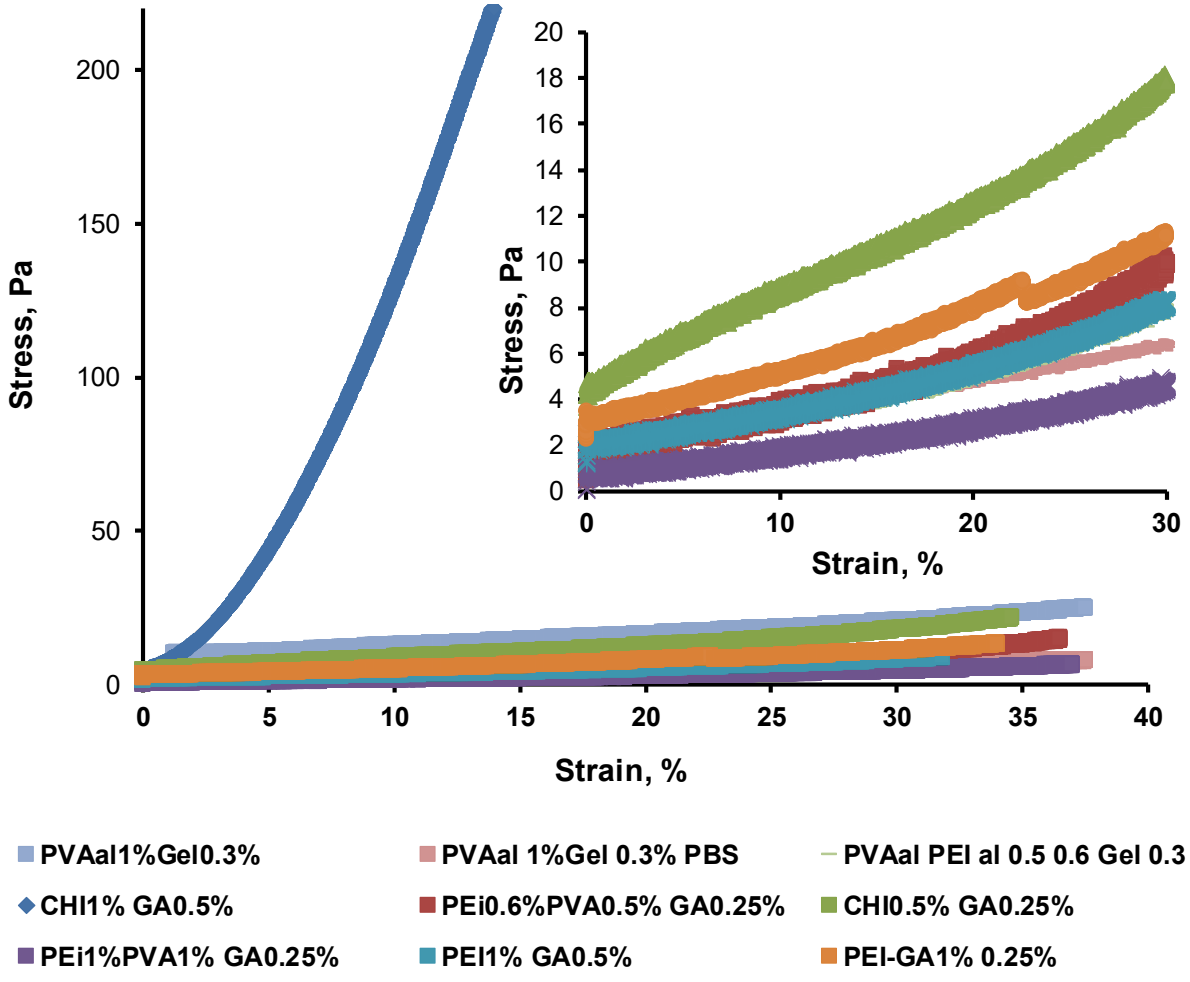

Fig. S4. Plot of elastic properties of composite cryogels based on *Rhodoccoccus korensis* using different polymer compositions and cross-linking agents.

148 **Table S1.** Evaluation of toxicity of polymers at different compositions and temperature, prior
149 to MTT assay incubation of bacteria (at 4 ºC for 24h, otherwise specified) of polymers with
150 PVA-al and PEI-al 0.6:0.6% (1:1).

Sample name	% of viable cells	Number of viable cells, CFU xE6
Pse & PBS (positive control)	50.0	62.5±1.4
Pse & PBS and 2% glucose.	43.0	53.9±1.15
Cryo-Pse-PEI-al:PVA-al-glucose 0.6:0.6:2.0(%); -12ºC	13.7	17.2±5.4
Cryo-Pse-PEI-al:PVA-al 0.6:0.6(%); -12ºC	12.0	15.0±0.25
Pse & PEI-al:PVAal 0.6:0.6(%)	34.4	43.2±5.4
Pse & PEI-al:PVA-al-glucose 0.6:0.6:2.0(%)	32.6	40.9±4.58
Pse & PBS initial (positive control)	100.0	125.53±2.8
Pse & PBS and 2% glucose initial	86.2	108.25±2.3
Rho & PBS (positive control)	100.0	802±15
Rho & PEI-al:PVA-al 0.6:0.6% and 0.9% NaCl	96.2	772.3±41
Rho & PVA-al 2.4%	76.5	613.8±14
Rho & PVA-al 3%	72.4	581±7
Rho & PEI 1.0% GA 0.5%(6000 CFU initially)	20.3	163±3.4
Rho & 0.5% GA in PBS (6000 CFU initially)	17.1	137.4 ±5
Acn & PEI-al 3%	75.2	44.6 ±1.1
Acn & PVA-al 2.4%	69.5	41.2 ±0.41
Acn & PEI 1% PBS	49.7	29.5 ±0.86
Acn & PEI 1% PBS 0.25% GA	13.7	8.12 ±0.15
Acn & GA 0.25% PBS negative control	7.6	4.54 ±0.22
Acn & PBS buffer positive control	100.0	59.3 ±1.2
Acn & PEI-al 0.365%	25.12	14.9 ±0.3
Acn & PVA-al 1.2%	92.4	54.8 ±0.23

Acn & PEI-al 0.5% PBS	13.8	8.2 ±0.2
Cryo-Acn-PEI-al-PVA-al 0.6:0.6%; -12C	24.3	14.4 ±1.9
Cryo-Acn-PEI-al-PVA-al-glucose 0.6:0.5:2.0%; -12C	17.4	10.3 ±0.74
Cryo-Acn-PVA-al-glucose 1.2:2.0(%) PBS; -12C	109.1	64.7 ±0.97
Acn & PVA-al-glucose 1.2:2.0%, PBS	54.13	32.1±7.9
Acn & PEI-al: PVA-al0.6:0.6%, PBS	44.5	26.4±0.9

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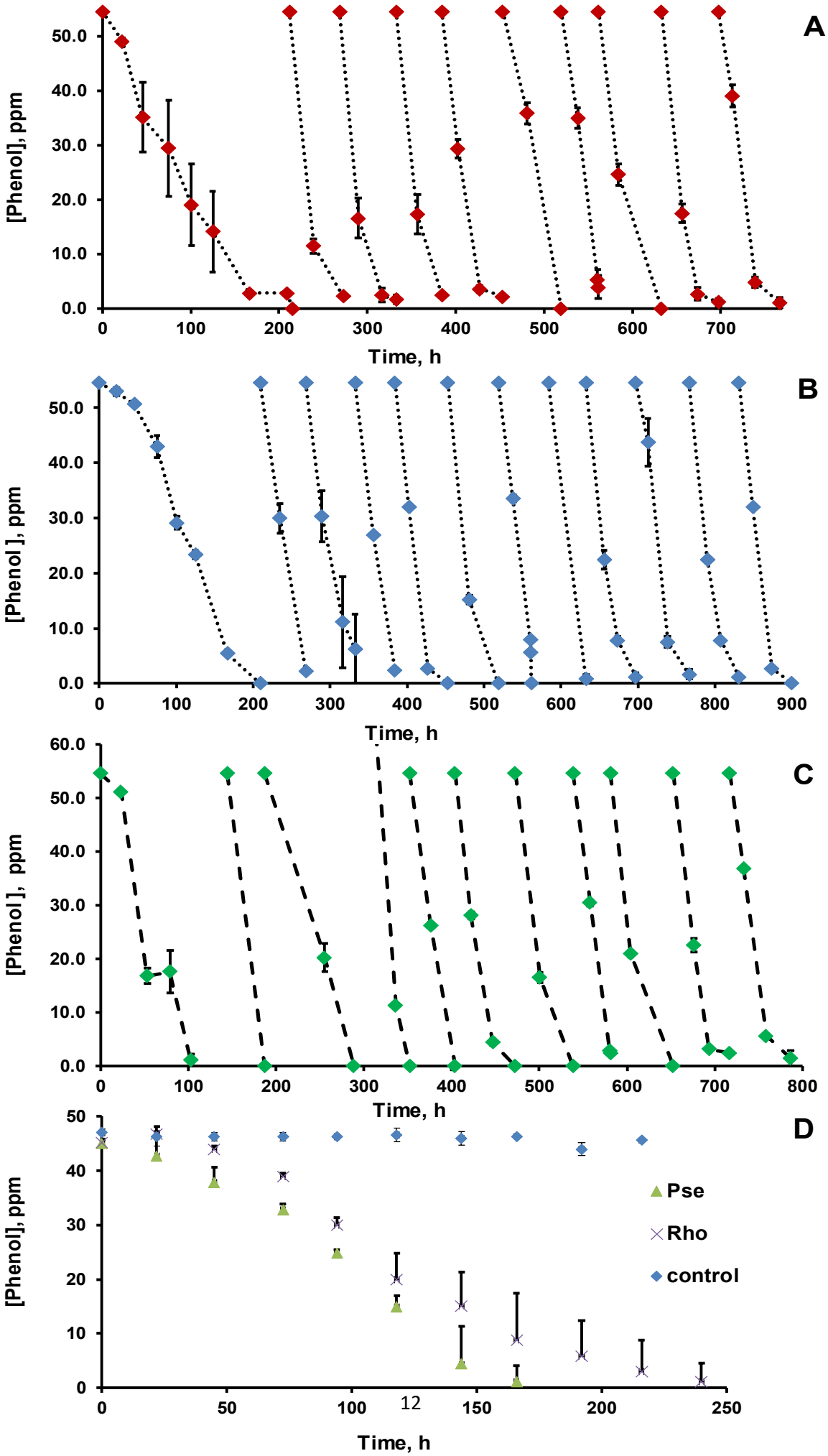

A
[Phenol], ppm
Time, h
B
[Phenol], ppm
Time, h
C
[Phenol], ppm
Time, h
D
[Phenol], ppm
Pse
Rho
control
Time, h

Fig. S5. Degradation of phenol by: **A)** CBR-*Acn*, **B)** CBR-*Pse*, **C)** CBR-*Rho* (2 plastic carriers (12.2±3.6x10^6 CFU) per each bottle V 200 mL; 54 ppm for 10 cycles, (n=3). **D)** Control experiment bioremediation of phenol(50ppm) in presence of suspension of bacteria *Pseudomonas mendocina* (▲), *Rhodococcus korensis* (x), solution of phenol without bacteria(♦), for 1st cycle (amount of bacteria comparable to amount of bacteria used in cryogels)(n=2).

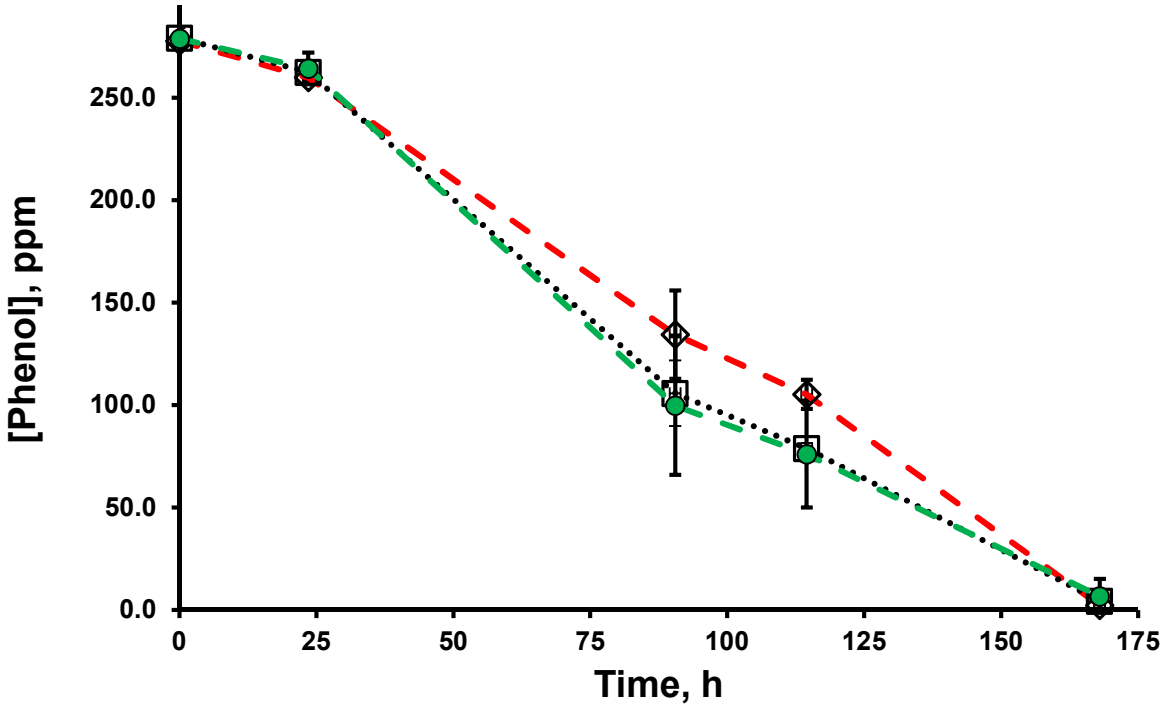


Fig. S6. Degradation of phenol in MSM vs time by: CBR-*Pse* (38±8.3x10^6 CFU)(●); CBR-Rho (576±52x10^6 CFU)(◊); CBR-Acn (17.6±0.96x10^6 CFU)(□), for 5th cycle, 2 plastic carriers per bottle V 200 mL, 280ppm phenol;

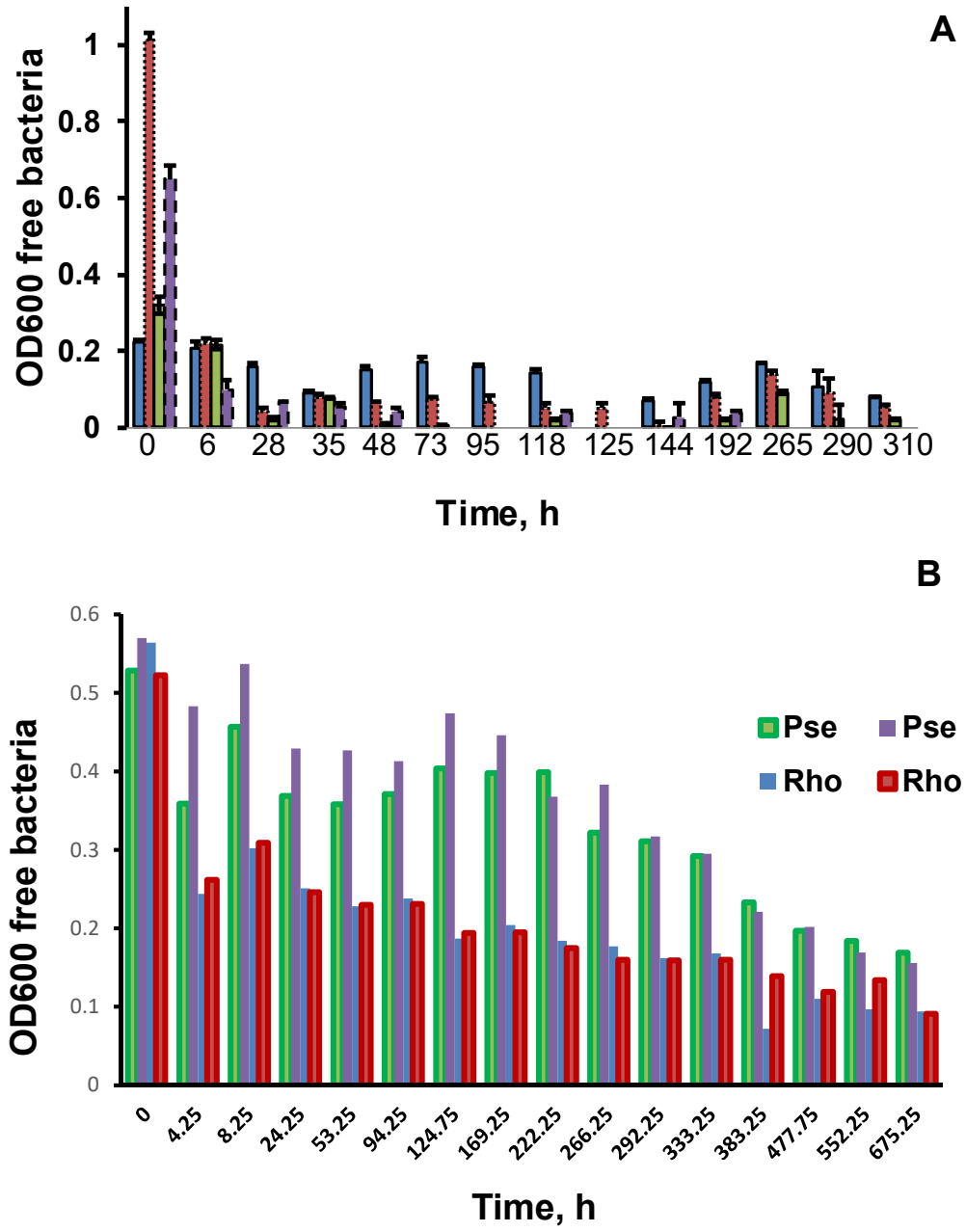


Fig. S7. Dynamics of change of viable bacteria of *Pse* (blue and green) and *Rho* (red and violet) in the 50 ppm 4-CP solution in a dynamic mode (shaking at 150 rpm) in buffer: A) 25 mM carbonate buffer; B) in MSM, (n=3).

Table S2. Concentration of 4-CP estimated using HPLC after the bioremediation period. Initial concentration of the 4-CP was 62 ppm, volume 40 mL.

Name of the sample	Remaining phenol after one month bioremediation, ppm	remaining phenol from initial, %
Rho suspension, **MSM** buffer	45.2	72.9
Control PEIal-PVA-al cryogel (no bacteria) **MSM** buffer	59.0	95.1
Control PVA-al cryogel (no bacteria) **MSM** buffer	61.31	98.9
Cryogel *Pse* PVA-al PEI-al 0.5% 0.6% **MSM**	34.84	56.2
Cryogel *Pse* PVA-al PEI-al 1.0%: 0.25% **MSM**	31.85	51.3
Cryogel *Pse* PVA-al 1% **MSM buffer**	25.95	41.8
Rho suspension, carbonate buffer	59.26	95.5
Rho suspension, carbonate buffer	61.07	98.3
Rho suspension, carbonate buffer	59.63	96.2
Pse suspension(commercial) carbonate buffer	55.76	89.9

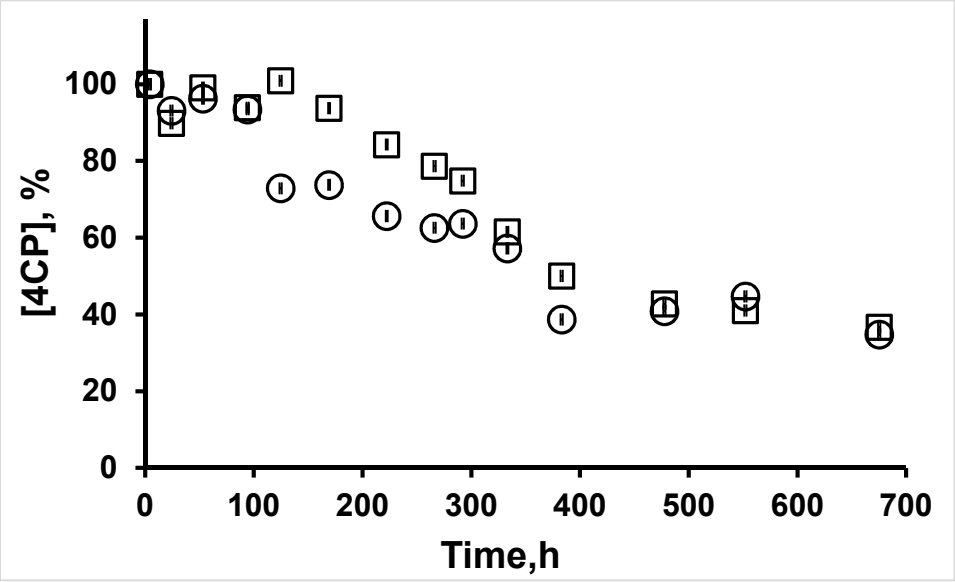
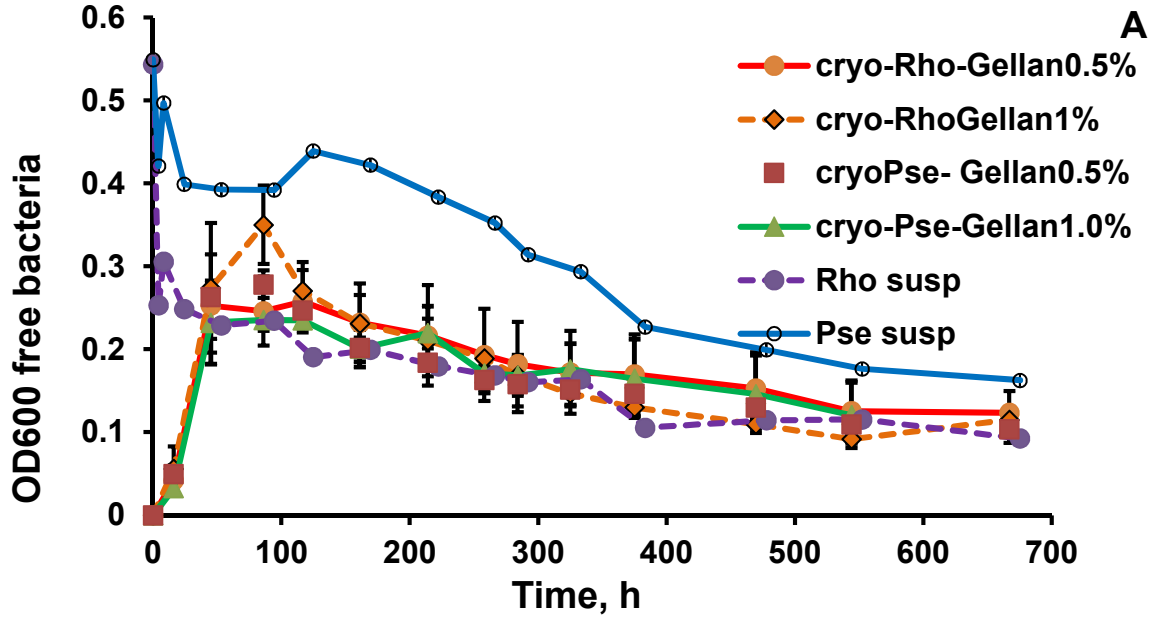
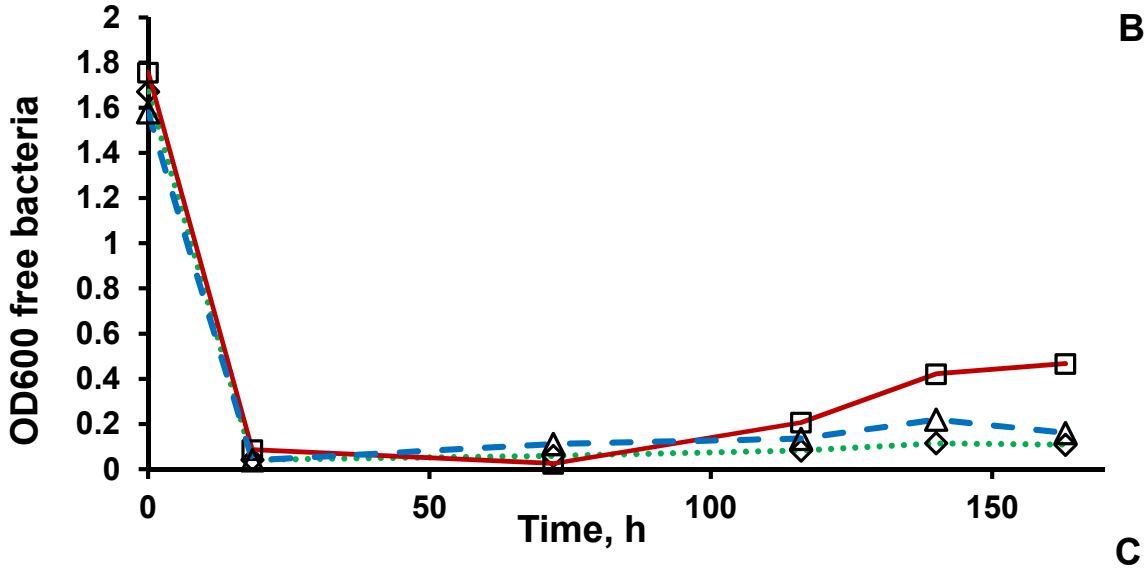


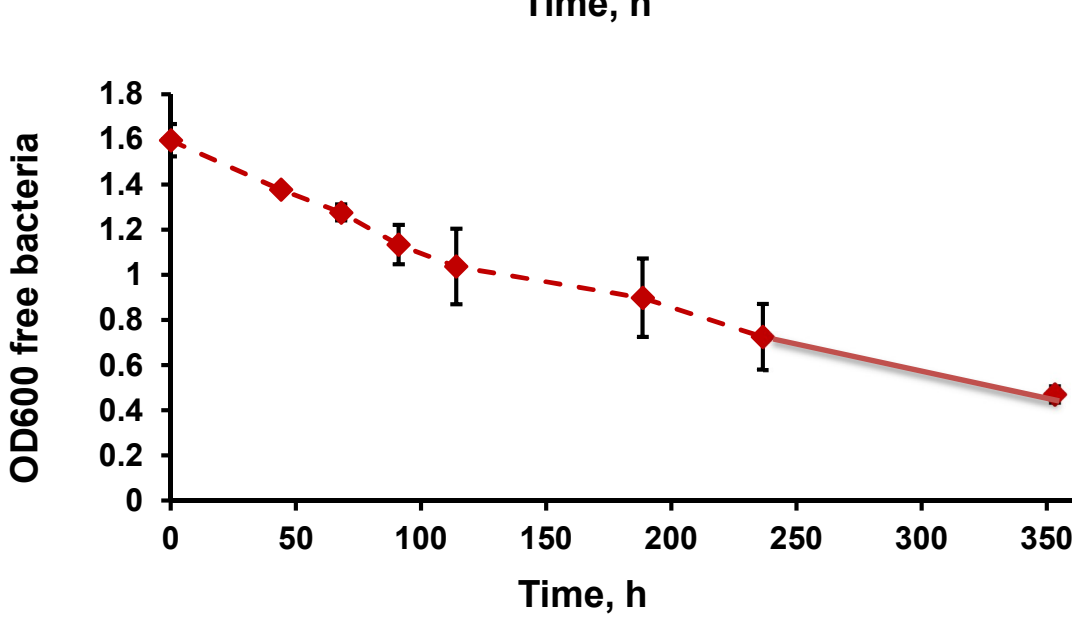
Fig. S8. Degradation of 4-CP (V 40 ml, 50 ppm) in MSM by suspension of free *Pse* 4-CP adapted (□), *Rho* 4-CP adapted (O) vs time in a dynamic mode (shaking at 150 rpm), number of cell $0.6x10^8$ per sample (n=2), concentration estimated using aminoantipyrine assay.


A
OD600 free bacteria
Time, h
cryo-Rho-Gellan0.5%
cryo-RhoGellan1%
cryoPse- Gellan0.5%
cryo-Pse-Gellan1.0%
Rho susp
Pse susp
0
0.1
0.2
0.3
0.4
0.5
0.6
0
100
200
300
400
500
600
700

177


B
OD600 free bacteria
Time, h
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
0
50
100
150

178


C
OD600 free bacteria
Time, h
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
0
50
100
150
200
250
300
350

179

Fig. S9. Dynamics of change of the population of free bacteria during the bioremediation process in a dynamic mode (shaking 150 rpm) : A) 50ppm of 4-CP solution in MSM; B) nonadapted on the agar plate, cultivated suspension *Rho* in MSM: 2-CP 60 ppm (▲); 2-CP 20 ppm (■); 4-CP 10 ppm (♦) (n=3). **C)** *Rho* adapted to CP 25 ppm) during the bioremediation process of 2-CP in MSM in a shaking mode (n=2).

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