Supplementary Information

One-pot chemo-enzymatic synthesis and one-step recovery of length-variable long-chain polyphosphates from microalgal biomass

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Supplementary Experimental Section

Free energy calculation

The Gibbs free energy for each biochemical reaction was computed utilizing the eQuilibrator tool (https://equilibrator.weizmann.ac.il/) [1]. The Gibbs free energies of the reactions at physiological standards ($\Delta rG'^m$) were approximated with the following parameters: reactant concentrations of 1 mM, ionic strength of 0.25 M, 1.0 mM Mg^{2+}, pH at 7.5, and a temperature of 25°C. The eQuilibrator API was used to estimate reaction Gibbs energies ($\Delta G'$) at an ionic strength of 0.1 M and 30°C, employing varying conditions as delineated below. For the creatine phosphorylation reaction, initial estimation of $\Delta G'$ was carried out across different pH values (7.0–10.0) and ATP concentrations (0.1–5.0 mM) under the specified experimental conditions: creatine at 15 mM, ADP at 0.15 mM, creatine phosphate at 15 mM, and Mg^{2+} at 10 mM. Subsequently, the impact of diverse Mg^{2+} concentrations (0.1–100 mM) on $\Delta G'$ for the creatine phosphorylation reaction was evaluated under varying pH conditions (4.0–10), while retaining ATP at 10 mM. Furthermore, the influence of varying creatine concentrations (0.1–50 mM) on the $\Delta G'$ of the creatine phosphorylation reaction was assessed at pH 9.0, using differing creatine phosphate concentrations (5.0 or 10 mM), and maintaining the following experimental conditions: Mg^{2+} at 10 mM, ATP at 0.7 mM, and ADP at 0.3 mM. For the reverse reaction (creatine de-phosphorylation), $\Delta G'$ was determined at pH 7.0 along with varying ATP concentrations (0.1–10 mM), under the specified experimental conditions: Mg^{2+} at 10 mM, ADP at 0.3 mM, creatine at 5 mM, and creatine phosphate at 0.8 mM.

Protein Expression and Purification

The genes encoding recombinant *Ralstonia* PPK2c were cloned into a pET28a(+) plasmid by IdealGene Biotech, Ltd. (Taichung, Taiwan) with codon optimization for *E. coli* heterologous expression (**Table S1**). The target gene sequences were linked to an N-His$_6$-tag using the Ser-Glu-Ala-Pro peptide linker. The *Cytophaga* PPK2 was amplified from the corresponding gDNA and then cloned into a pMCSG19 plasmid as described previously (**Table S1**) [2]. The *E. coli* strain JW2487 expressing exopolyphosphatase (PPX) was purchased from the ASKA collection (NBRP, SHIGEN, Shizuoka, Japan) and rabbit muscle creatine kinase was purchased from Roche (Basel, Switzerland) (**Table S1**). For standard expression and purification of recombinant enzymes, recombinant *E.coli* BL21(DE3) cultures were cultivated under aerobic conditions at 37°C in a shaker containing Terrific Broth at 200 rpm until a specific optical density was reached (OD$_{600 nm}$ 0.6). Protein overexpression was induced with isopropyl β-D-1-thiogalactopyranoside (0.4 mM), and the culture was then incubated overnight at 16°C while rotating at 200 rpm. Recombinant proteins were purified to at least 95% homogeneity using immobilized metal affinity chromatography (Ni Sepharose High-Performance resin) as previously described [3]. In brief, cells were collected by centrifugation at 8,000 × g for 10 min at 4°C and resuspended in binding buffer (HEPES (4-(2-hydroxyethyl)-L-piperazineethanesulfonic acid)-Na buffer (pH 7.5; 25 mM), NaCl (0.3 M), glycerol (5%, w/w), and imidazole (5 mM)). The cells were then homogenized *via* sonication in an ice bath for 10 min (1 s on and 2 s off). The lysate was centrifuged at 20,000 × g for 20 min at 4°C and the supernatant was passed through a glass gravity column containing 0.5 mL Ni Sepharose^{TM} High-Performance resin (GE Healthcare, Chicago, IL, USA). After washing with wash buffer (HEPES-Na buffer (pH 7.5; 25 mM), NaCl (0.3 M), glycerol (5%, w/w), and imidazole (25 mM)), proteins were eluted with 0.4 mL of elution buffer (HEPES-Na buffer (pH

7.5; 25 mM), NaCl (0.3 M), glycerol (5%, w/w), and imidazole (300 mM)). Protein purity was then analyzed by SDS-PAGE (**Appendix**). The protein stock solutions were flash-frozen in liquid nitrogen, stored at −80°C, and thawed immediately before use. The N-His$_6$ tag was retained for all experiments. All subsequent enzyme assays were conducted in duplicate or triplicate as indicated in each respective figure. The enzyme kinetics were used to determine the assay conditions (**Table S2**).

Electrophoretic analysis of polyP using TBO-stained TBE-Urea polyacrylamide gel electrophoresis

Electrophoretic analysis of polyP was performed following an established protocol described previously [4]. The reaction mixtures containing polyP were pre-mixed with 2x sample buffer (2x Tris-Borate-EDTA (TBE), urea (7 M), and bromophenol blue (0.1 g/L)) and loaded onto a separating gel consisting of urea (7 M), acrylamide (6%, v/v), and 1x TBE. Electrophoresis was performed in a 1x TBE buffer bath for 25 min at 200 V at room temperature (25°C). The gel was stained in TBO solution containing urea (0.05%, w/v), methanol (25%, v/v), and glycerol (5%, w/w) for 10 min, and was de-stained for 1 h in an identical solution but without TBO. After de-staining, the magenta-colored bands on the TBE-Urea gel represent TBO-stained polyP. The size range of the polyP was estimated by comparison to polyP standards (chain lengths of 14, 60, 130, and 200–500 (average chain length: 243); provided by Toshikazu Shiba of RegeneTiss).

High-Performance Liquid Chromatography (HPLC) analysis

Quantification of creatine phosphate and adenosine triphosphate (ATP) by HPLC was performed following a protocol described previously with some modifications [5]. Briefly, the separation of creatine phosphate and ATP in the reaction mixture was performed on a 150 × 4.6 mm Luna®

C18(2) 100 Å, LC column packed with 5-μm particles (Phenomenex, USA) while simultaneously recording $\lambda_{210\ nm}$, indicative of creatine phosphate. The acquired $\lambda_{210\ nm}$ values were later converted to creatine phosphate concentration based on a standard curve derived from the different concentrations of the corresponding chemicals. The mobile phase consisted of buffer A (Na_2HPO_4 (20 mM)) and buffer B (Na_2HPO_4 (200 mM) and acetonitrile (10%, v/v)), all adjusted to pH 5.0 with phosphoric acid (2 M) and filtered through a 0.22-μm membrane filter. The total regeneration time for the HPLC column is 20 min. Aliquots of 10 μL were injected into the instrument by autosampler, and the subsequent elution was performed at a flow rate of 0.4 mL/min in buffer A for 2 min, followed by a 1:1 gradient elution with buffers A and B up to 8.5 min, and additionally with buffer B up to 10 min. The column was cleaned by washing with 80% methanol after each run for 10 min and was re-equilibrated with buffer A for 1 min.

Enzymatic digestion/elongation of polyP using Cytophaga PPK2/Ralstonia PPK2c *Cytophaga* PPK2 and *Ralstonia* PPK2c were used to degrade and elongate commercial polyP (~25-mer), respectively. The degradation of polyP was conducted within reaction mixtures (200 μL) containing HEPES-K (pH 7.5; 100 mM), Mg^{2+} (5 mM), polyP (~25-mer; 1 mM), ADP (8 mM), and *Cytophaga* PPK2 (0.1 mg/mL), while polyP elongation (200 μL) was conducted in reaction mixtures with ATP (8 mM) and *Ralstonia* PPK2c (0.5 mg/mL). The reactions were initiated by the addition of *Cytophaga* PPK2/*Ralstonia* PPK2c at 30°C. The length of the resulting polyP was monitored by TBE-Urea polyacrylamide gel electrophoresis.

Determination of the length distribution of the generated polyP 1,300-mer

Toshikazu Shiba of RegeneTiss (Kunitachi, Tokyo, Japan) performed gel filtration chromatography *via* HPLC, using EX-polyP (available from RegeneTiss) of various sizes (14-mer,

60-mer, and 130-mer) as a standard, as according to experimental details described in previous publications[6]. We similarly applied polyacrylamide gel electrophoresis using the same standards to confirm the length of the homogeneous long-chain polyP products in parallel.
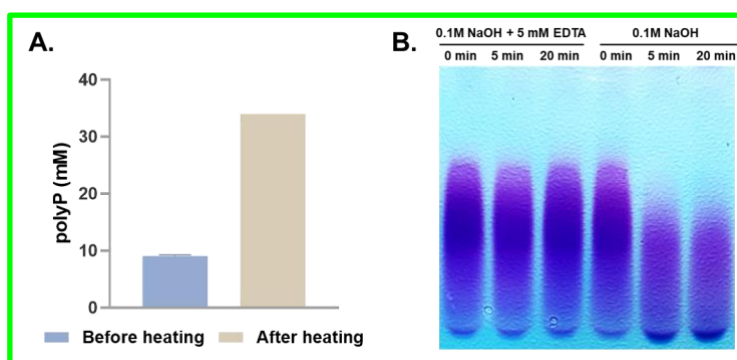
Supplementary Figures.

Figure S1. The effect of the boiling step in the extraction of algal polyP on the extracted polyP quantity and its length distribution. Related to Fig. 2. **(A)** Algal polyP was extracted with and without the boiling step (100°C, 10 min). The concentrations of the extracted algal polyP were measured by TBO assay. **(B)** 100-mer polyP was incubated in a buffer containing 0.1 M NaOH with or without 5 mM EDTA. The reaction mixtures from different time points were analyzed by TBE-Urea polyacrylamide gel electrophoresis.
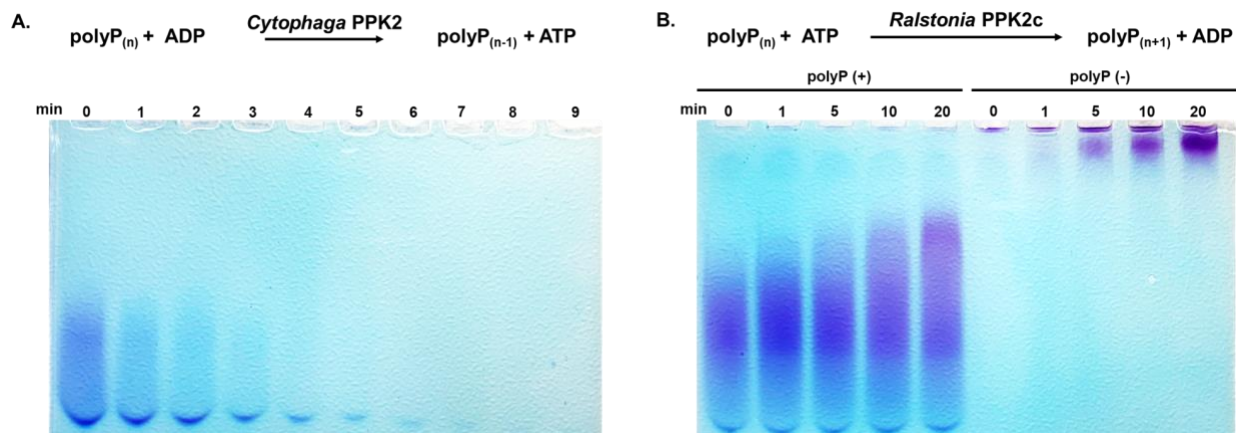
Figure S2. Electrophoretic analysis of time-dependent polyP 25-mer mixture degradation or elongation using *Cytophaga* PPK2 or *Ralstonia* PPK2c, respectively. Related to Figs. 3–5. **(A)** *Cytophaga* PPK2 and **(B)** *Ralstonia* PPK2c were used for the degradation or elongation of the commercial polyP 25-mer. The reaction mixtures from different time points were analyzed by TBE-Urea polyacrylamide gel electrophoresis.
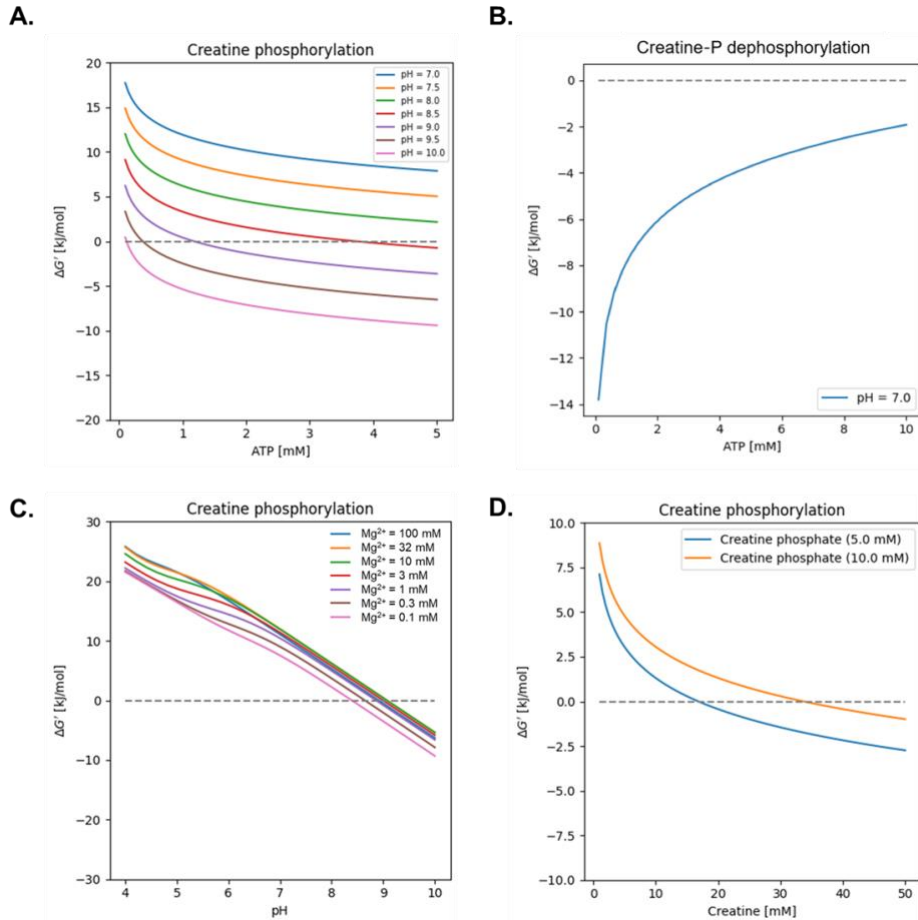
Figure S3. eQuilibrator-based estimation of the reaction Gibbs free energy (ΔG′) of the enzymatic reactions. Related to Figs. 4 and 5. **(A)** The ΔG′ of the creatine phosphorylation reaction was estimated at different pH and concentrations of ATP under the following experimental conditions: ionic strength (0.1 M), creatine (15 mM), ADP (0.15 mM), creatine phosphate (15 mM), Mg^{2+} (10 mM), and 30°C. **(B)** The ΔG′ of the creatine phosphate dephosphorylation reaction was estimated at pH 7.0 along with different concentrations of ATP under the following experimental conditions: ionic strength (0.1 M), Mg^{2+} (10 mM), ADP (0.3 mM), creatine (5 mM), creatine phosphate (0.8 mM), and 30°C. **(C)** The ΔG′ of the creatine phosphorylation reaction was estimated at different pH and concentrations of Mg^{2+} under the following experimental conditions: ionic strength (0.1 M), creatine (15 mM), ADP (0.15 mM), and creatine phosphate (15 mM), ATP (10 mM), and 30°C. **(D)** The ΔG′ of the creatine phosphorylation reaction was estimated at pH 9.0 at different concentrations of creatine phosphate along with different concentrations of creatine under the following experimental conditions: ionic strength (0.1 M), Mg^{2+} (10 mM), ATP (0.7 mM), ADP (0.3 mM), and 30°C.
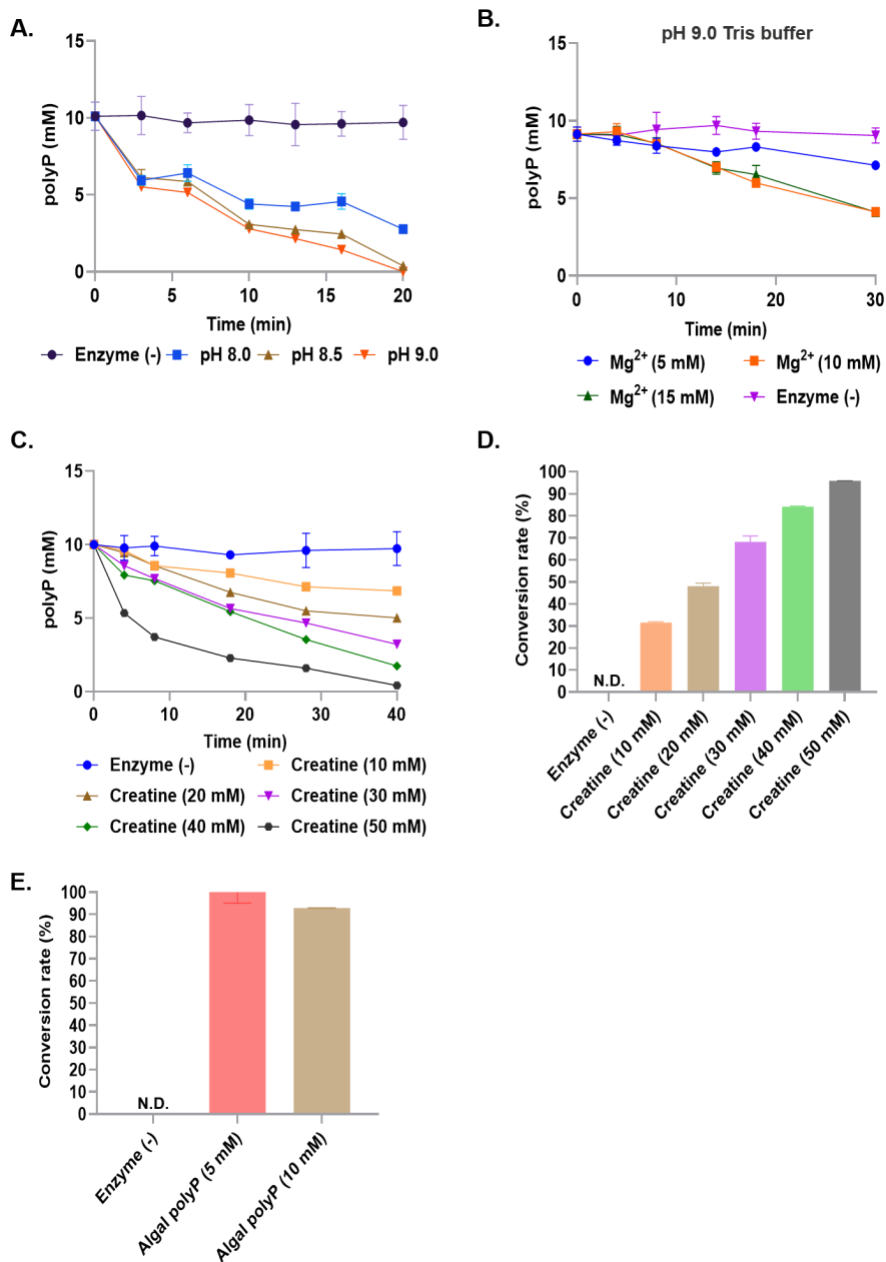
8

Figure S4. Optimization of the CK-PPK2c cascade reaction for creatine phosphate production from the algal polyP and creatine. Related to Fig. 4. Creatine phosphate production by the PPK2-CK cascade was evaluated based on polyP consumption. The PPK2-CK cascade reactions were conducted at different pH (**A**), different concentrations of Mg^{2+} (**B**), and different concentrations of creatine (**C**) in Tris (pH 9.0). The conversion rates of polyP to creatine phosphate in the reaction mixture with different concentrations of creatine (**D**) and the algal polyP (**E**) were determined using HPLC. Error bars represent means ± SEM from independent experiments in triplicate.

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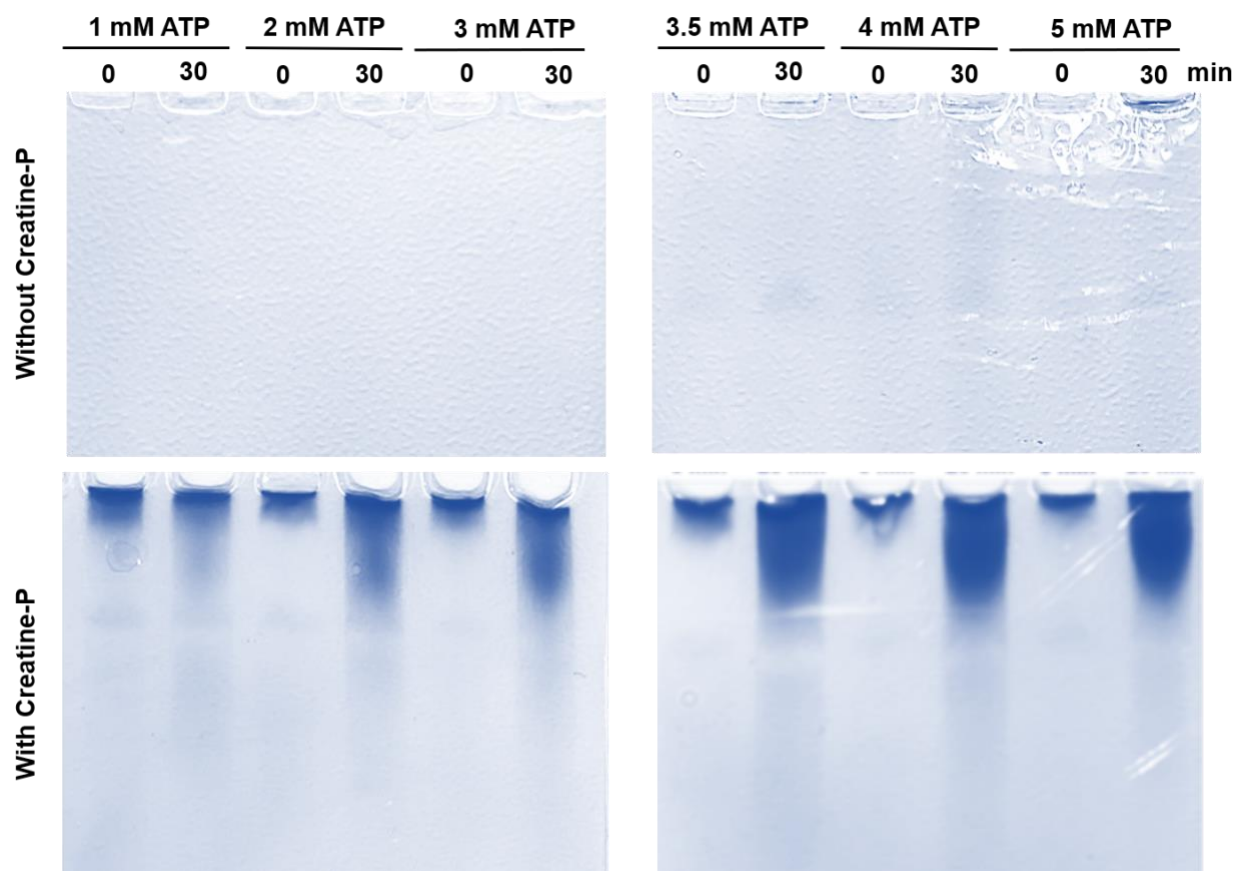
Figure S5. TBE-Urea polyacrylamide gel electrophoresis analysis of the *Ralstonia* PPK2c-catalyzed synthesis of the polyP 1,300-mer using creatine-P, along with varying ATP concentrations, as the P-donor. Related to Fig. 5. The reaction was conducted with and without creatine phosphate, along with varying concentrations of ATP.
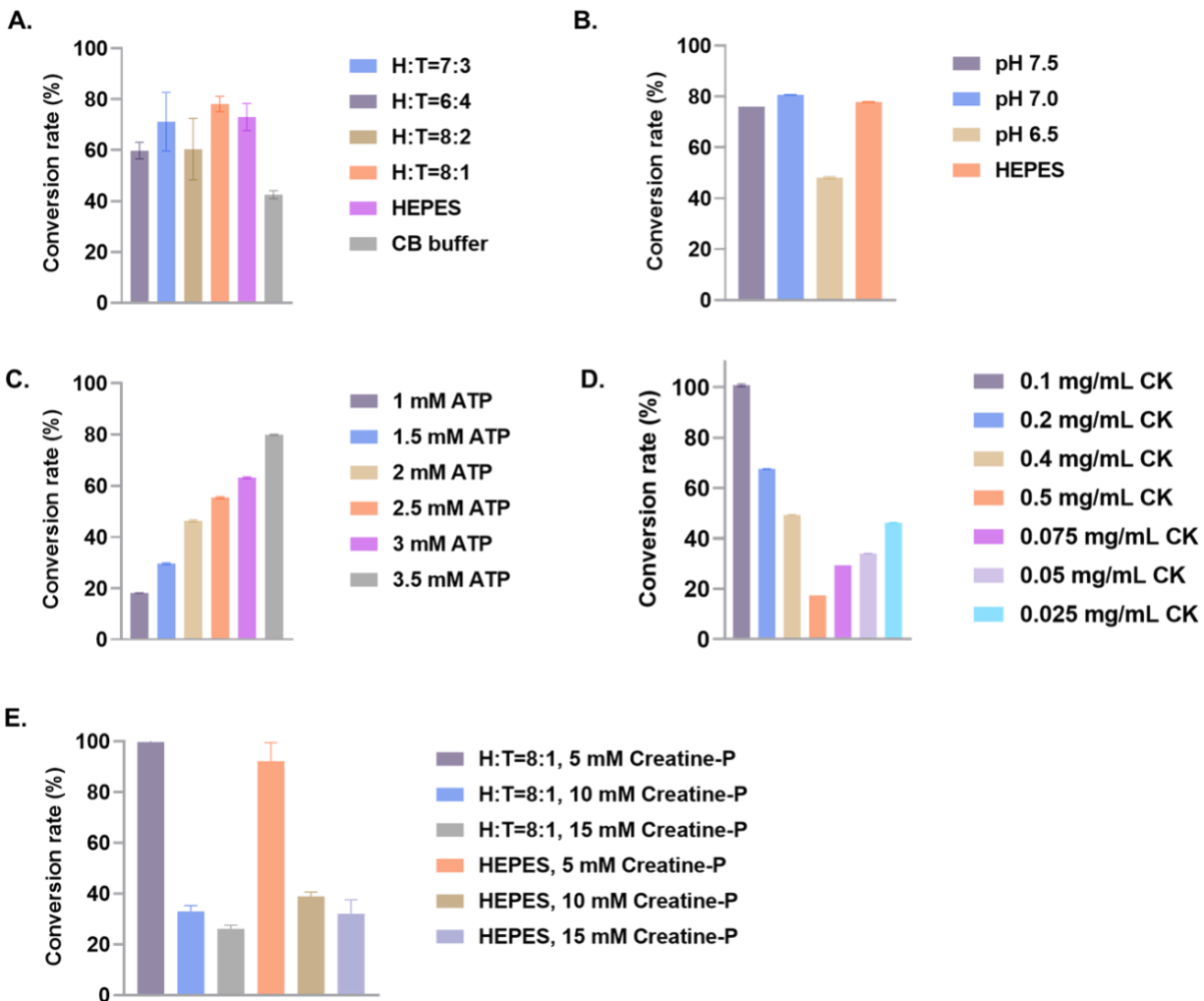
Figure S6. Optimization of the CK-PPK2c cascade reaction for the one-pot, two-step enzymatic synthesis of insoluble long-chain polyP. Related to Figs. 5 and 6. The CK-PPK2c cascade reactions were conducted in the mixed buffers with different ratios of HEPES and Tris (**A**). The same cascade reaction was then conducted in 8:1 HEPES:Tris at different pH (along with reactions in HEPES at pH 7.0) (**B**) and at different concentrations of ATP (**C**), CK (**D**), and creatine phosphate (along with reactions in HEPES at the same creatine phosphate concentrations) (**E**). The conversion rates of creatine phosphate to polyP in the CK-PPK2c cascade reaction under different conditions were determined using high-performance liquid chromatography. Values represent means ± SEM from independent experiments in triplicate.
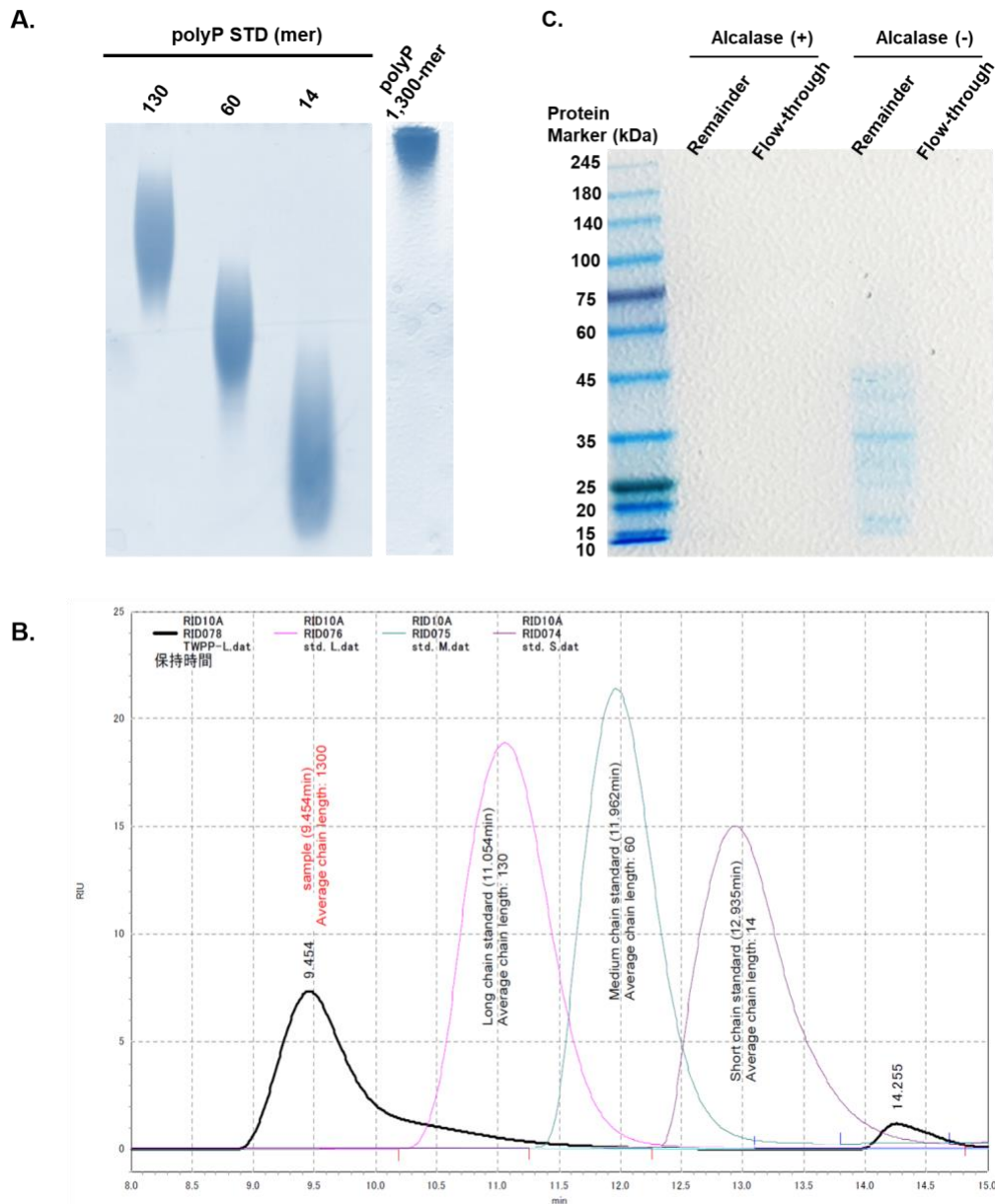
Figure S7. Purification and determination of the length of the insoluble polyP product. Related to Fig. 7. The reaction mixture containing the insoluble long-chain polyP was filtrated before and after protolysis treatment. **(A)** The insoluble long-chain polyP within the remainder fraction was then analyzed by TBE-Urea polyacrylamide gel electrophoresis, along with commercial polyP standards at various lengths (14-mer, 60-mer, 130-mer) as a standard to determine polyP product length. **(B)** The average length of the insoluble long-chain polyP was also determined *via* HPLC along with the same commercial polyP standards as standards to determine the product polyP length to be ~1,300-mer. **(C)** Removal of proteins from the remainder fraction following purification was verified by SDS-PAGE analysis.
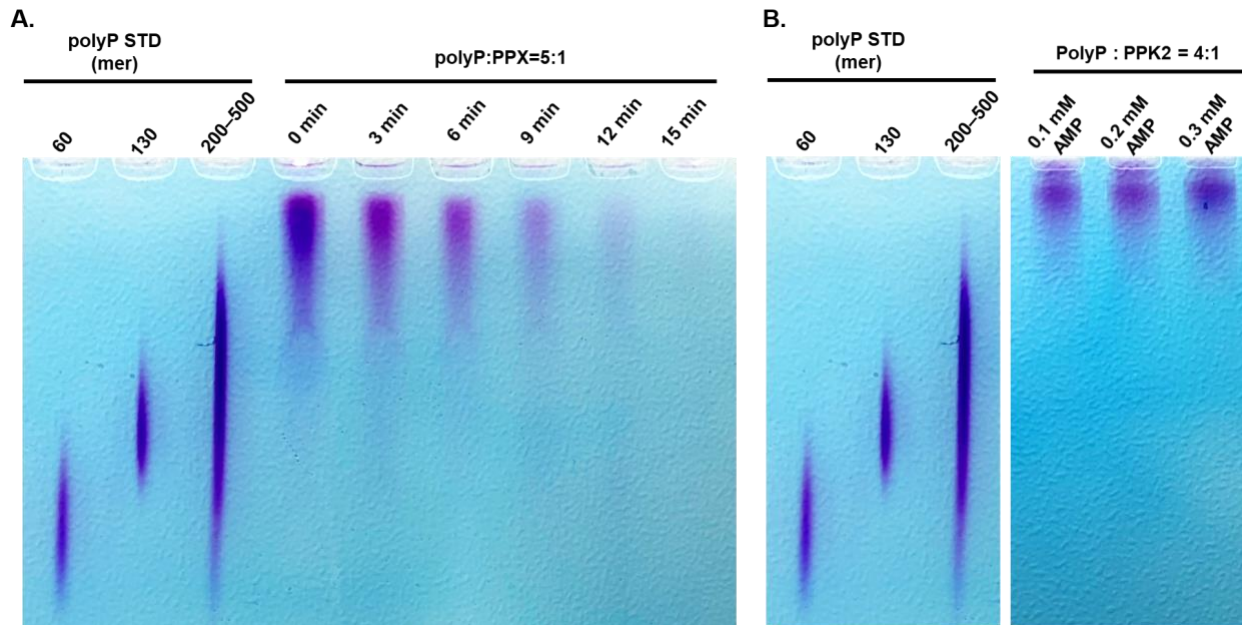
Figure S8. Time-dependent enzymatic digestion of homogeneous long-chain polyphosphate (polyP) 1,300-mer using PPX or PPK2. Related to Fig. 8. The polyP 1,300-mer was incubated with **(A)** PPX or **(B)** PPK2 to digest the polyP. The polyP and PPX or PPK2 were mixed at ratios of 5:1 or 4:1, respectively. The reaction mixtures collected at different time points were analyzed by TBE-Urea polyacrylamide gel electrophoresis, along with the commercial polyP standards of varying lengths as a reference for the lengths.

Supplementary Tables

Table S1. Complete list of recombinant proteins and expression vectors used in this work.

Enzyme	Abbreviation	Molecular weight (kDa)	UniProt accession	Host organism
Cytophaga PPK2	PPK2	72	A0A6N4SMB5	*Cytophaga hutchinsonii*
Ralstonia PPK2c	PPK2c	42	Q0KCB8	*Ralstonia eutropha*
Creatine kinase	CK	43	P00567	*Oryctolagus cuniculus*
Exopolyphosphatase	PPX	58	P0AFL6	*Escherichia coli*

Table S2. K_m and k_{cat} of the enzymes used in this work (the values were extracted from the BRENDA database)

Enzyme	Substrate	K_m (mM)	k_{cat} (1/s)	k_{cat}/K_m (1/s·mM)
Cytophaga PPK2	Polyphosphate	0.3 ± 0.1	30.0 ± 5	1.0×10^5
	AMP	0.6 ± 0.10	17.0 ± 1	2.7×10^4
	ADP	0.4 ± 0.1	9.5 ± 0.5	2.7×10^4
	ATP	2.7 ± 0.7	0.3 ± 0.1	1.0×10^2
Ralstonia PPK2c	ATP	4.5 ± 2	10.0 ± 2	2.0×10^3
Creatine kinase	ADP	0.3	N.A.	N.A.
	ATP	0.7	N.A.	N.A.
	Creatine	5.0	N.A.	N.A.
	Creatine phosphate	0.8	N.A.	N.A.
Exopolyphosphatase	N.A.	N.A.	N.A.	N.A.

Table S3. Carbon footprint of the traditional and the novel routes for polyP synthesis

Traditional Methods

	Temperature	Carbon footprint
Producing a >500-mer polyP	775°C for 1 h	$\frac{(775-30)\times4.2}{1\times3,600}\ kWh\ \times\ 0.5\ kg\ CO_2/kWh\ =\ 0.435\ kg\ CO_2$
Producing a <500-mer polyP	700°C for 1 h	$\frac{(700-30)\times4.2}{1\times3,600}\ kWh\ \times\ 0.5\ kg\ CO_2/kWh\ =\ 0.391\ kg\ CO_2$

Novel method in this study

|		Temperature	Carbon footprint
Producing polyP 1,300-mer	*C. vulgaris* polyP extraction	100°C for 10 min (⅙ h)	
	Enzymatic synthesis of creatine phosphate from polydisperse polyP in microalgal cell-lysate	30°C	$\frac{[(100-30)+(30-30)+(30-30)]\times4.2}{6\times3,600}\ kWh\ \times\ 0.5\ kg\ CO_2/kWh$ $=0.007\ kg\ CO_2$
	Enzymatic synthesis of homogeneous polyP 1,300-mer	30°C	
Producing a polyP 100-mer	Degradation of homogeneous polyP 1,300-mer by non-enzymatic hydrolysis	95°C for 1 h	$\frac{(95-30)\times4.2}{1\times3,600}\ kWh\times0.5\ kg\ CO_2/kWh\ =\ 0.038\ kg\ CO_2$
Total carbon footprint			$0.007\ kg\ CO_2\ +\ 0.038\ kg\ CO_2\ =\ 0.045\ kg\ CO_2$

1 kWh = 3600 kJ × 1h = 0.5 kg CO_2 （Tsai & Kuo, 2010）
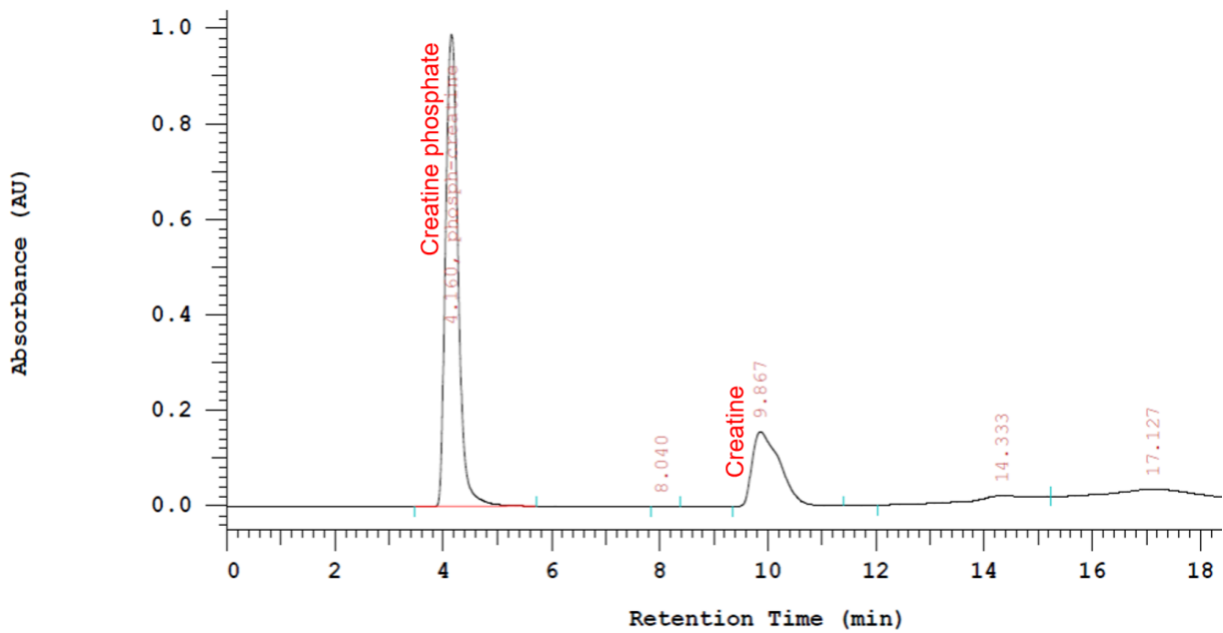↑1°C/ 1 kg water = 4.2 kJ
* Assuming a complete heat insulation of the reactors without heat loss.

Appendix

SDS-PAGE gel images of purified *Cytophaga* class III PPK2 (~72 kDa), *Ralstonia* PPK2c (~42 kDa), rabbit creatine kinase (CK; ~43 kDa), and exopolyphosphatase (PPX; ~58 kDa).
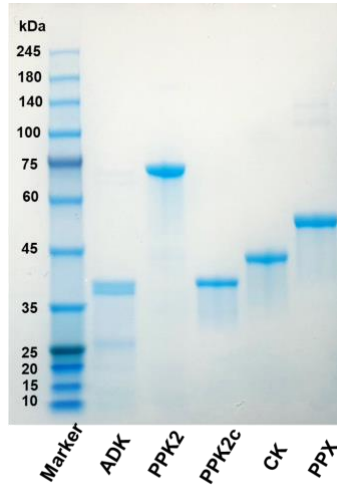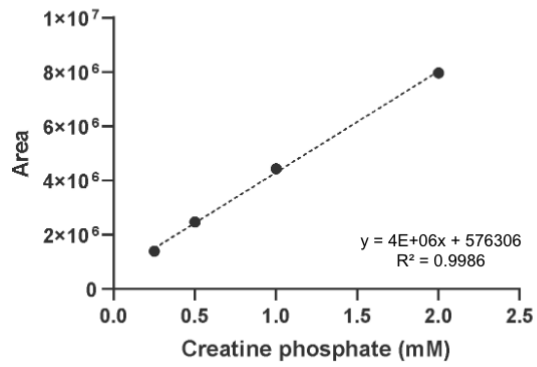

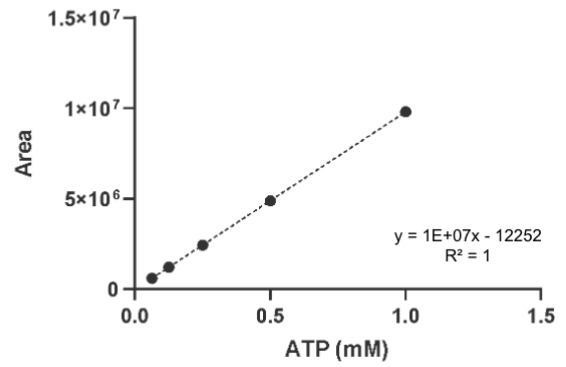**HPLC chromatograms of commercial creatine phosphate (4.1 min), and creatine (10.0 min) standards.**
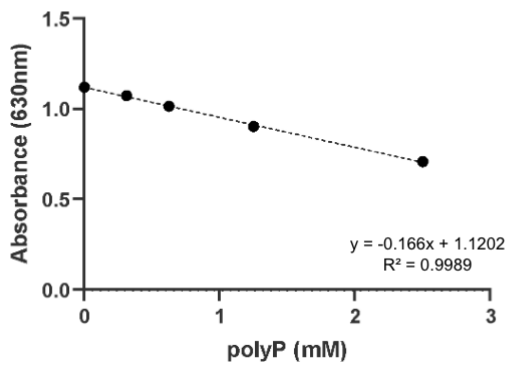
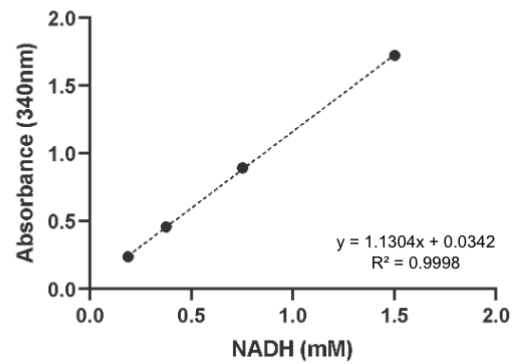**Optical standard curves used in this study**

Standard curve of creatine phosphate

$$y = 4E{+}06x + 576306$$
$$R^2 = 0.9986$$

Standard curve of ATP

$$y = 1E{+}07x - 12252$$
$$R^2 = 1$$

Standard curve of TBO assay

$$y = -0.166x + 1.1202$$
$$R^2 = 0.9989$$

Standard curve of NADPH

$$y = 1.1304x + 0.0342$$
$$R^2 = 0.9998$$

The P-rich wastewater-sampling site
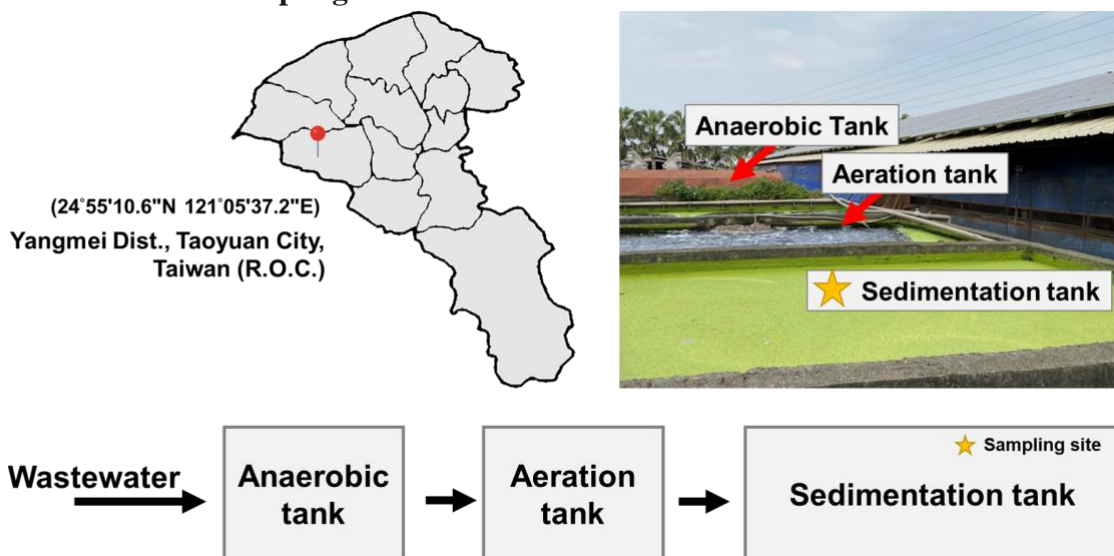
Supplementary References

1   M. E. Beber, M. G. Gollub, D. Mozaffari, K. M. Shebek, A. I. Flamholz, R. Milo and E. Noor, *Nucleic Acids Research*, 2022, **50**, D603–D609.

2   B. P. Nocek, A. N. Khusnutdinova, M. Ruszkowski, R. Flick, M. Burda, K. Batyrova, G. Brown, A. Mucha, A. Joachimiak, Ł. Berlicki and A. F. Yakunin, *ACS Catalysis*, 2018, **8**, 10746–10760.

3   E. Kuznetsova, M. Proudfoot, C. F. Gonzalez, G. Brown, M. V. Omelchenko, I. Borozan, L. Carmel, Y. I. Wolf, H. Mori, A. V. Savchenko, C. H. Arrowsmith, E. V. Koonin, A. M. Edwards and A. F. Yakunin ,*Journal of Biological Chemistry*, 2006, **281**, 36149–36161.

4   P.-H. Wang, K. Fujishima, S. Berhanu, Y. Kuruma, T. Z. Jia, A. N. Khusnutdinova, A. F. Yakunin and S. E. McGlynn, *ACS Synthetic Biology*, 2020, **9**, 36–42.

5   P. Sikk, T. Käämbre, H. Vija, K. Tepp, T. Tiivel, A. Nutt and V. Saks, *Proceedings of the Estonian Academy of Sciences*, 2009, **58**, 122.

6   H. Itoh and T. Shiba, *Journal of Bacteriology*, 2004, **186**, 5178–5181.