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Biocatalytic Cascade of Polyphosphate Kinase and Sucrose Synthase for Synthesis of Nucleotide-activated Derivates of Glucose

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1. Biocatalytic Cascade of Polyphosphate Kinase and Sucrose Synthase for Synthesis of Nucleotide-activated Derivatives of Glucose

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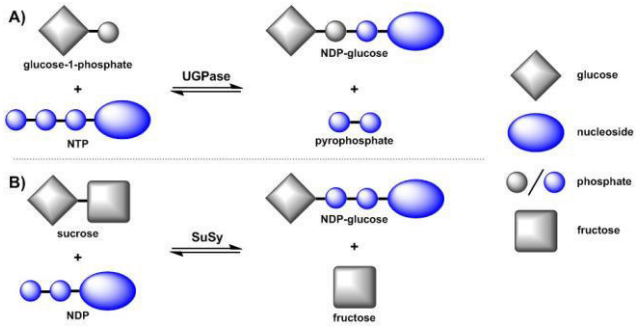
Abstract. Sucrose synthase (SuSy) catalyzes conversion of sucrose in the presence of a pyrimidine or purine nucleoside diphosphate (NDP) to the corresponding nucleotide-activated derivative of glucose (NDP-glucose). To realize the potential of SuSy for NDP-glucose synthesis fully, a nucleoside monophosphate (NMP) should be employed in the reaction, for it is the much more cost-effective substrate than NDP. The use of class II/III polyphosphate kinases (PPK), catalyzing conversion of NMP into NDP in the presence of polyphosphate (polyP), was therefore explored in this study and a biocatalytic cascade of PPK (from *Meiothermus ruber*) and SuSy (from *Acidithiobacillus caldus*) for NDP-glucose production was established. The synthetic efficiency of the cascade reflected the NMP substrate specificity of the PPK, following the order AMP > GMP > CMP > UMP > dTMP. It was also influenced by the concentrations of Mg^{2+} and polyP as well as by the pH. An optimized synthesis at 45°C and pH 5.5 gave 70 mM (41 g L^{-1}) of ADP-glucose from 100 mM of AMP and 132 mM of polyP in the presence of excess of sucrose (1 M) and using Mg^{2+} (25 mM). The productivity was ~14 g (L^{-1} h^{-1}) despite low enzyme concentrations used in the reaction (≤ 300 µg mL^{-1}). Isolation of ADP-glucose (~99% purity) by anion-exchange chromatography required prior removal of the polyphosphate, which we showed to be achievable by fractional precipitation with ethanol. The herein developed coupling with PPK, to form the NDP substrate from NMP in situ, could be generally useful to advance NDP-sugar synthesis by Leloir glycosyltransferases.

Keywords: NDP-glucose; biocatalysis; glycosylation; phosphorylation; nucleotides

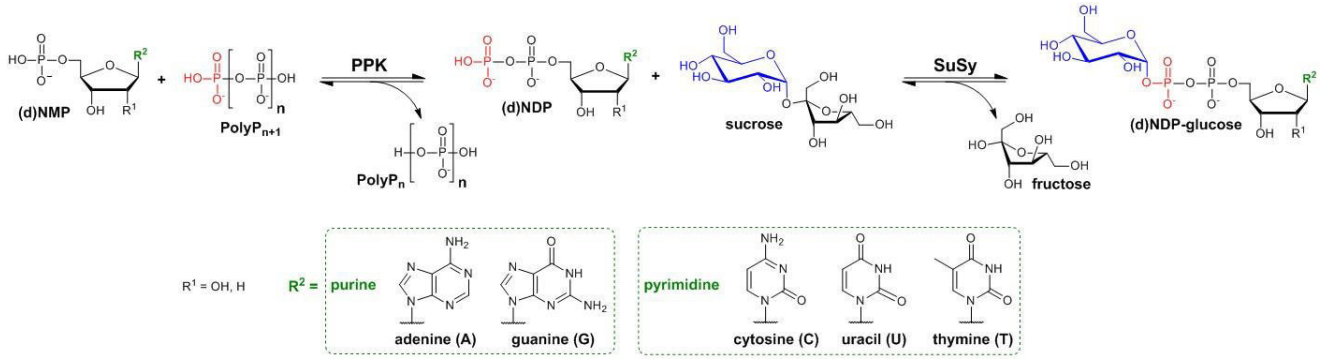
1.1. Introduction

For common sugars, such as glucose, galactose, mannose and several others, nucleoside diphosphate (NDP)-derivatives represent the naturally activated molecular forms used in cellular biosynthesis.[1] NDP-sugars are the substrates of Leloir glycosyltransferases, a large class of enzymes catalyzing the glycosidic attachment of sugars during assembly of a variety of glycostructures, including oligo- and polysaccharides as well as glycosylated natural products, glycoproteins and glycolipids.[2] NDP-sugars are important high-value biochemicals and reagents with broad use in glycobiology, to assay glycosyltransferase activity for example,[3] as well as in the synthesis of speciality carbohydrates.[2c, 4] NDP-sugars are prepared naturally, and also in biomimetic synthesis, through transformations by multi-enzyme cascades.[1a, 5] Typically sugar phosphorylation from a nucleoside triphosphate (NTP) is followed by nucleotidyl transfer from a second NTP (Scheme 1A).[6] The reverse reaction of Leloir glycosyltransferases represents an alternative direct (single-step) route towards NDP-sugars (Scheme 1B). A suitable glycoside serves as the "donor" substrate converted in the presence of NDP.[7] Clear advantage of the "glycosyltransferase route" is its simplicity, for only a single enzyme is used, and therefore its relative ease of optimization. However, a donor sufficiently high in energy to drive glycosylation of the NDP is required, but convenient availability of such a one can be a problem.

In the synthesis of NDP-activated forms of glucose, sucrose appears to be an ideal donor, for it comprises an exceptionally energy-rich disaccharide structure and is furthermore a highly expedient substrate.[7b, 8] Work of Elling and colleagues has shown that sucrose synthase (SuSy; EC 2.4.1.13) utilizes different NDP acceptors (uridine 5'-diphosphate, UDP; adenine 5'-diphosphate, ADP; guanosine 5'-diphosphate, GDP; cytosine 5'-diphosphate, CDP; deoxythymidine 5'-diphosphate, dTDP) to convert sucrose into the corresponding NDP-glucoses, therefore raising considerable interest in the synthetic use of this enzyme.[9] More recently, we have shown that, based on detailed evaluation of kinetic and thermodynamic constraints of the biocatalytic process, performance metrics of UDP-glucose synthesis with



Scheme 1. Enzymatic synthesis of NDP-glucose either involves UDP-glucose pyrophosphorylase (A) or SuSy (B).



Scheme 2. One-pot synthesis of NDP-glucose starts from the inexpensive substrates NMP, polyP and sucrose. NMP is phosphorylated by PPK to NDP, which is then glucosylated by SuSy.

SuSy could be pushed to meet the typical requirements of fine chemicals production in the industry.[10] However, it was also recognized that the high potential of SuSy could be realized far more effectively, if instead of the costly NDP substrate the much more inexpensive nucleoside 5'-monophosphate was employed in the reaction.[7b, 9b] Based on current reagent prices, the economic benefit of using NMP compared to NDP could be as high as 100-fold. In a study of gram-scale production of ADP-glucose by SuSy, Elling used adenylate kinase (E.C 2.7.4.3)-catalyzed conversion of AMP and ATP to prepare the ADP substrate in situ.[9b] However, the nucleoside phosphates were utilized only partially in process that is furthermore restricted to adenosine phosphates and requires ATP. Therefore, the formation of NDP via direct enzymatic phosphorylation of NMP from the expedient phospho-donor polyP seemed to be a more promising approach. The present study describes development of a cascade of polyphosphate kinase (PPK) and SuSy for efficient synthesis of NDP-glucoses (Scheme 2).

PPKs are categorized according to sequence similarity into two types, 1 and 2.[11] Within the type 2 group of enzymes, PPKs are further subdivided according to specificity for the substrate, NDP (class I), NMP (class II) or both (class III), phosphorylated from polyP.[11-12] Type 1 PPKs use ATP to produce polyphosphate.[13] For the purpose of this study, only type 2 PPKs of class II and III were suitable, and a member of each class was therefore examined.

Reports about the application of PPKs in biocatalysis are relatively scarce and their use was so far restricted mainly to regenerate ATP from ADP.[14] Applying the phosphorylation-nucleotidyl transfer route (Scheme 1) to the synthesis of UDP-galactose,[14a, 15] UDP-glucose[15] and CMP-*N*-acetylneuraminic acid,[16] the PPK-polyphosphate system was used to form the ATP required for enzymatic phosphorylations of UMP and UDP or that of cytosine. Up to 7 enzymes were needed in multienzyme cascade transformations performed in one pot.[14a] Therefore, in order to telescope the *formation* and the *glucosylation* of NDP into a single overall conversion (Scheme 2), the NDP should be directly produced from NMP, which in the context of a biocatalytic cascade is reported here for the first time. Five NDP derivatives of glucose (ADP, UDP, GDP, CDP, dTDP) were synthesized using coupled PPK and SuSy. Optimized production at high yield and productivity was demonstrated for ADP-glucose.

1.2. Results and Discussion

All experiments were performed with affinity-purified enzymes obtained from *Escherichia coli* overexpression cultures, as described in the Supporting Information (Figure S1). Enzyme activities were determined using a reversed-phase ion-pairing HPLC assay (Figures S2-6).

1.2.1. Selecting a PPK for NMP phosphorylation

The type 2 PPKs from *Acinetobacter johnsonii* (class II; *Aj*PPK)[17] and *Meiothermus ruber* (class III; *Mr*PPK)[12] were compared. Basic biochemical properties of the two PPKs were known from earlier papers,[12, 17] but their utility in forming NDP from NMP needed to be assessed. Figure 1 shows results of phosphorylation of different NMP substrates. Both enzymes preferred reaction with the purine-type NMPs (AMP, GMP). Among the pyrimidine NMPs, CMP was preferably used. Only small activities were observed with UMP and dTMP, especially when using *Aj*PPK which was inactive with UMP. Consistent with their class II or III placement, *Aj*PPK only formed NDP as product while *Mr*PPK continued phosphorylation on NDP to also synthesize NTP.[11-12] The relative amount of NTP in total product varied with the NMP substrate used. It was particularly significant in the reaction of AMP. Overall, *Mr*PPK appeared to be better suited to provide a broad range of NDPs. Partial conversion of NDP to NTP was potentially problematic, for it would compete with the SuSy reaction. However, we show later that advantageously it did not interfere with the NDP-glucose production.

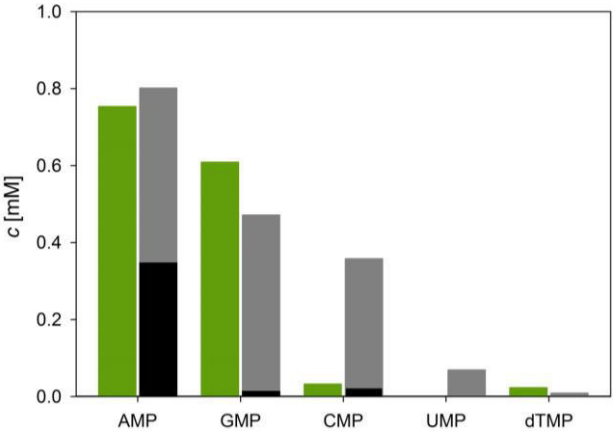


Figure 1. Phosphorylation of different NMP acceptors by *Aj*PPK (green, NDP) and *Mr*PPK (grey, NDP; black, NTP) is shown. Reaction conditions: 1 mM NMP, 6.6 mM polyP, 50 µg mL^{-1} PPK, pH 7.2, 6 h, 30°C.

We considered that accumulation of high amounts of NDP-glucose in simple batch conversions necessitates that the PPK used tolerate the required high concentrations of polyP in the reaction. Results in Figure S7 show that both PPKs were inhibited at elevated polyP levels, however, *Mr*PPK much less so than *Aj*PPK. At 100 mM polyP, ADP formation with *Mr*PPK was more than 10 times faster than it was with *Aj*PPK. Furthermore, because of the strong pH dependence of its reaction equilibrium, SuSy cannot be applied above pH 5.5.[10] However, *Aj*PPK was reported to be inactive at pH 5.5 and lower.[17a] It was shown here that *Mr*PPK was stable at pH 5.5 and lost its activity only below pH 4.0. Finally, the far superior thermostability was recognized as another advantage of *Mr*PPK.[12, 17a] In conclusion, therefore, *Mr*PPK was the enzyme of choice to be used in combination with SuSy.

1.2.2. Establishing the biocatalytic cascade

To select a SuSy suitable for coupling with *Mr*PPK, we evaluated an enzyme from *Glycine max* (soybean; *Gm*SuSy)[18] and another one from *Acidithiobacillus caldus* (*Ac*SuSy),[19] representing the two main evolutionary lineages of SuSy which are in plants and in bacteria. Both SuSys have previously been characterized in the synthesis of UDP-glucose from sucrose and UDP.[10] Coupled reactions to convert AMP (10 mM) into ADP-glucose were performed applying *Mr*PPK (200 µg mL^{-1}) in 4-fold excess over SuSy so as to make the SuSy activity (partly) limiting overall. PolyP (33 mM) and sucrose (250 mM) were applied in excess to drive the product formation. Because *Gm*SuSy is less thermostable than *Ac*SuSy and *Mr*PPK,[10, 12] the reaction of *Gm*SuSy was done at 30°C, that of *Ac*SuSy at 45°C. Time courses of ADP-glucose production are compared in Figure 2. Although ADP-glucose synthesis was feasible with both SuSys it was much faster when *Ac*SuSy was used. To release ~6 mM ADP-glucose took about 10 min in the *Ac*SuSy reaction while it took about 5 h in the *Gm*SuSy reaction.

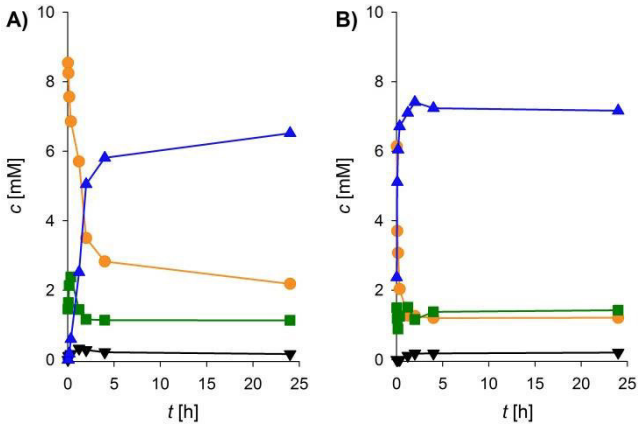


Figure 2. Time course analysis of ADP-glucose synthesis from AMP using *Mr*PPK in tandem with *Gm*SuSy (A) and *Ac*SuSy (B) at 30 and 45°C, respectively, is shown. Reaction conditions: 10 mM AMP, 10 mM $MgCl_2$, 33 mM polyP, 250 mM sucrose, 200 µg mL^{-1} *Mr*PPK, 50 µg mL^{-1} SuSy, pH 5.5. AMP (orange), ADP (green), ADP-glucose (blue), ATP (black).

The difference in rate of the SuSy reactions was also reflected in the course of the ADP concentration. Using *Gm*SuSy, ADP accumulated initially to a concentration of ~2.4 mM, only to decrease afterwards to a stable concentration of around 1.4 mM. Using *Ac*SuSy, by contrast, the ADP remained constant at ~1.4 mM throughout the whole conversion. Note that only ~0.2 mM ATP was formed in both reactions, clearly indicating that AMP "overphosphorylation" by *Mr*PPK was not a problem. The marked benefit in terms of productivity obtained from the coupling of *Mr*PPK with *Ac*SuSy, as compared to the coupling with *Gm*SuSy, was likely the combined result of the common preference of *Mr*PPK and *Ac*SuSy for the nucleobase adenine (as compared to the preference of *Gm*SuSy for uridine) and the higher applicable reaction temperature.[12, 18b, 19]

In a next step we evaluated the substrate scope of the *Mr*PPK-*Ac*SuSy cascade reaction. Yields after 24 h are shown in Figure 3 and the time courses of NMP conversion can be found in Figure S8. All NMPs were successfully converted to the corresponding NDP-glucoses but there were marked differences in the reaction rates. As anticipated from the substrate preference of *Mr*PPK (Figure 1), production of ADP-glucose and GDP-glucose was favored. However, CDP-glucose and UDP-glucose were also synthesized in significant amounts. Even dTMP-glucose was formed to an appreciable extent. We note, and shown in Figure S8, that the ADP-glucose rate exceeded that of GDP-glucose by at least 7-fold, although the finally obtained GDP-glucose (8.1 mM) was slightly higher than ADP-glucose (7.7 mM). Based on NDP-

glucose synthesis rates, therefore, the substrate preference of the cascade conversion was identical to that of the individual *Mr*PPK reaction (Figure 1), following the order AMP > GMP > CMP > UMP > dTMP. In all conversions shown in Figure 3, less than 1 mM NDP accumulated during the reaction course. Therefore, this suggested that *Mr*PPK was the limiting enzyme in the biocatalytic cascade under the conditions used, despite the fact that it was applied in six-fold excess (by mass) over *Ac*SuSy.

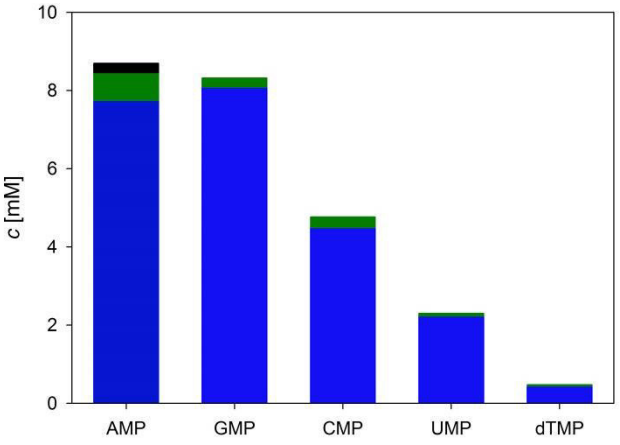


Figure 3. Final conversion of 10 mM NMP by 300 µg mL^{-1} *Mr*PPK and 50 µg mL^{-1}*Ac*SuSy after 24 h incubation at 45°C (33 mM polyP, 250 mM sucrose, pH 5.5). NDP (green), NDP-glucose (blue), NTP (black).

To assess the synthetic potential of the cascade reaction and optimize its use for NDP-glucose production, we further on focused on the synthesis of ADP-glucose.

1.2.3. Optimized synthesis of ADP-glucose

Economic constraints of the fine chemicals production usually dictate that biocatalytic conversions not only utilize inexpensive substrates but also accumulate high final product concentrations, typically 50 g L^{-1} or more.[20] To obtain such high titers of NDP-glucose, at least 100 mM of NMP substrate needed to be applied in the reaction. Effects of the pH and of the concentrations of $MgCl_2$ and polyP had to be assessed carefully to enable an efficient conversion under these conditions.

Figure 4A shows that low concentrations of $MgCl_2$ (≤ 25 mM) stimulated the ADP-glucose synthesis whereas higher concentrations resulted in inhibition. Additional problem at high Mg^{2+} was gradual precipitation of the polyP. Evidence that ADP also increased at high Mg^{2+} (Figure 4A) suggested that Mg^{2+} has a negative influence on the conversion as result of a thermodynamic effect. Studies of the closely related sucrose-phosphate synthase[21] support the notion that Mg^{2+} affects the equilibrium constant of the glucosyl transfer reaction. The role of Mg^{2+} is likely complex and it was beyond the scope of the current work to analyze it in detail. It seemed sufficient to have identified a 25 mM concentration of $MgCl_2$ as the optimum for ADP-glucose formation.

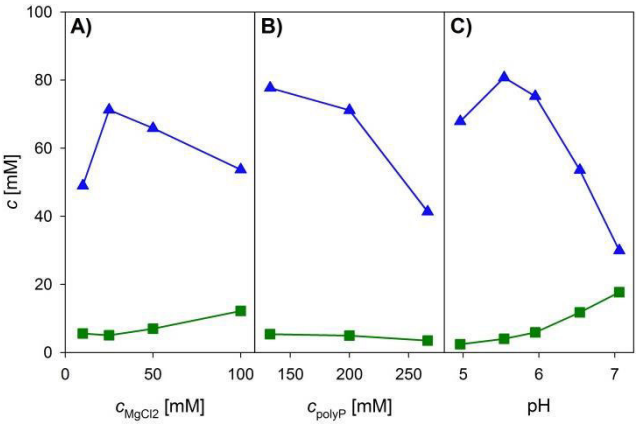


Figure 4. The effects of $MgCl_2$ (A) and polyP (B) concentration and pH (C) on ADP-glucose yields in the *Mr*PPK-*Ac*SuSy cascade reaction were analyzed. Standard reaction conditions: 100 mM AMP, 132 mM polyP, 1000 mM sucrose, 100 µg mL^{-1} *Mr*PPK, 50 µg mL^{-1} *Ac*SuSy, pH 5.5, 45°C, 48 (A) or 24 h (B, C). ADP (green), ADP-glucose (blue).

The polyP concentration was a key factor of reaction optimization, not only for its effect by mass action on the conversion of AMP but also because of its potential impact on PPK kinetics. Besides the substrate inhibition discussed earlier, *Mr*PPK was known to lose most of its activity when the length of the polyP substrate decreases below five phosphate units.[12] Figure 4B shows that the ADP-glucose titer dropped significantly on increasing the polyP concentration from 132 mM to 265 mM. The molar ratio of ADP-glucose and ADP in final reaction mixtures was however constant at a value of ~13, showing that *Ac*SuSy was not affected by the change in the polyP concentration. The trend of the results in Figure 4B implies that polyP chain length did not limit the ADP-glucose yield. The decrease in ADP-glucose formation at higher polyP concentration was probably obtained due to substrate inhibition of *Mr*PPK. To minimize this effect, a polyP concentration of 132 mM was used in all further conversions.

Finally, effect of the pH in the range 5.0 - 7.0 was examined (Figure 4C). The ADP-glucose titer dropped sharply above and below an optimum pH of 5.5. The cause for decreased product formation is different at high and low pH, as revealed clearly by comparing the levels of ADP-glucose and ADP in the final product mixture. The drop in the ADP-glucose concentration at high pH was paralleled by an increase in the ADP concentration, suggesting that the observed pH dependence reflects the influence of the pH on the equilibrium of the SuSy reaction. We have shown in a recent study of UDP-glucose production by SuSy that the conversion of sucrose

and UDP proceeded most efficiently at low pH (≤ 5.0) and that the pH dependence of the reaction equilibrium reflected the protonation of UDP below an apparent pK_a of around 6.[10] Figure S9 shows that ADP-glucose formation from ADP exhibited a similar pH dependence. The decrease in the ADP-glucose concentration at a pH smaller than 5.5 was due to a substantially lowered activity (≥70% loss) of the *Mr*PPK under these conditions, thus rendering it impossible to benefit from the favorable effect of further lowering the pH on the equilibrium of the SuSy reaction (Figure S9). In summary therefore, a pH of 5.5 represented the best compromise between a high reaction rate and a suitable reaction equilibrium.

Reaction conditions considered optimal from Figure 4 (25 mM $MgCl_2$, 132 mM polyP and pH 5.5) were applied to ADP-glucose synthesis from 100 mM AMP. ADP-glucose formation was characterized by rapid accumulation of product to a concentration of 69 mM (41 g L^{-1}) in the first 5 h of reaction. This corresponds to a space-time-yield (*STY*) of about 14 mM h^{-1} (8.1 g L^{-1} h^{-1}). The maximum amount of ADP-glucose released after 24 h was 81 mM (48 g L^{-1}), corresponding to a yield of 81% based on the AMP initially added to the reaction. Evidence that only little ADP accumulated during the conversion indicates its efficient utilization in the *Ac*SuSy reaction and shows that the overall conversion was limited by the *Mr*PPK reaction. The activity of *Ac*SuSy was sufficient to maintain the ratio of ADP-glucose to ADP throughout the conversion at an apparent equilibrium around 20:1. Even at the end of the conversion only ~4 mM ADP was present and phosphorylation of ADP to ATP was negligible (< 0.75 mM).

The herein described synthetic route represents a significant advancement over previous reports on ADP-glucose production.[9a, 9b, 22] Most notably, Elling and coworkers isolated 2.2 g of ADP-glucose from a coupled reaction of adenylate kinase (from rabbit muscle) and SuSy (from potato).[9b] However, main drawback of the approach was the moderate final ADP-glucose concentration of just 4.4 mM. Although AMP was used, the reaction still required ATP (4.0 mM) to form ADP and the yield based on total adenine phosphates added to the reaction was 55%. By substituting the adenylate kinase reaction with the PPK reaction we fully avoided use of expensive substrates like ATP or ADP and achieved significant boost in the ADP-glucose concentration (18-fold) and increased the yield by 26%. The final ADP-glucose concentration, 41 - 48 g L^{-1} depending on when one is prepared to stop the reaction in Figure 5, roughly matched the broadly defined target value for industrial fine chemical synthesis.[20] Similar product concentrations were obtained in biocatalytic syntheses of NDP-sugars according to the phosphorylation-nucleotidyl transfer route in Scheme 1: UDP-galactose (44 g L^{-1}),[23] CMP-*N*-acetylneuraminic acid (34 g L^{-1}),[16] UDP- glucose

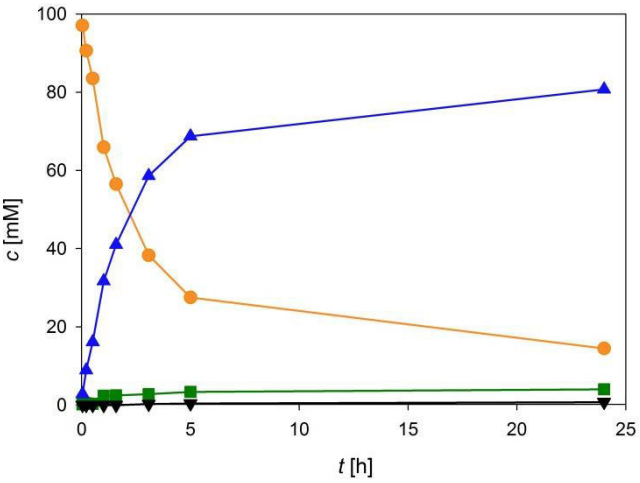


Figure 5. A time course of ADP-glucose synthesis by the *Mr*PPK-*Ac*SuSy cascade reaction under optimized conditions is shown: 100 mM AMP, 1000 mM sucrose, 132 mM polyP, 25 mM $MgCl_2$, pH 5.5. AMP (orange), ADP (green), ADP-glucose (blue), ATP (black).

(34 g L^{-1})[24] and GDP-fucose (18 g L^{-1}).[25] In the field of NDP-glucose production, the current synthesis was only outmatched by the direct conversion of sucrose and UDP into UDP-glucose (144 g L^{-1}) catalyzed by *Ac*SuSy, as recently reported by Gutmann and Nidetzky.[10] However, use of the costly UDP was a clear limitation. The *STY* of the *Mr*PPK-*Ac*SuSy reaction was 3-12-fold lower than that of the direct UDP-glucose synthesis (25 g L^{-1} h^{-1}),[10] but there was clearly a favorable trade-off between *STY* and substrate costs on moving from the direct conversion to the coupled reaction with PPK. The *STY* obtained here was comparable or exceeded the *STY*s from the alternative synthesis of UDP-glucose (4.3 g L^{-1} h^{-1})[24] or the syntheses CMP-*N*-acetylneuraminic acid (2.9 g L^{-1} h^{-1})[16] and UDP-galactose (2.1 g L^{-1} h^{-1}).[23] It should be note, however, that the current ADP-glucose synthesis was achieved with a low enzyme loading of only 50 µg mL^{-1} *Ac*SuSy and 100 µg mL^{-1} *Mr*PPK. Previous cascade reactions, by contrast, relied on high enzyme loading, typically in the form of whole cells (up to 215 g L^{-1}).[23-25] We therefore achieved an excellent mass-based turnover number (TTN_{mass} in g product/g enzyme) of 320 g g^{-1} which already approached the typical industrial requirement of 1000 g g^{-1}.[20] Altogether the *Mr*PPK-*Ac*SuSy cascade reaction represents a significant advance in ADP-glucose synthesis and emphasizes the large potential of PPKs to facilitate cost-efficient synthesis of further NDP-glucoses and NDP-sugars in general.

1.2.4. Establishing purification of ADP-glucose

NDP-sugars are rather unstable molecules which makes their isolation from reaction mixtures a problem requiring special attention.[26] Here in particular, it was important to evaluate the unknown

impact of polyP on the recovery of ADP-glucose. To obtain sufficient amounts of ADP-glucose, the enzymatic synthesis was performed in a 150-mL magnetically stirred reactor. Figure S10 shows that the reaction scale-up was possible in principle although the final yield was somewhat lower (68%) than in the small-scale conversion. A white precipitate was formed gradually during the conversion, primarily from the polyP but also from the adenine phosphates in the reaction mixture, and this eventually affected the final ADP-glucose titer (57 mM). One relevant factor might be the agitation mode which was changed on moving from the shaken tube to the stirred reactor. Critical parameters of reaction scale up may have to be evaluated systematically, but in view of the conceptual approach of the current study, this was left for consideration in the future. However, one immediate process solution would be to add the polyP in portions, thus avoiding precipitate formation.

Considering evidence from our previous study that phosphatase-catalyzed dephosphorylation of nucleoside phosphates followed by selective precipitation with ethanol constitutes a convenient method of chromatography-free isolation of NDP-glucoses from SuSy reaction mixtures,[26] we initially examined the same for the sample from the *Mr*PPK-*Ac*SuSy conversion. Unfortunately, the calf intestine phosphatase used was strongly inhibited by polyP so that even after prolonged incubation of 24 h large amounts of AMP and ADP remained intact (Figure S11). Another problem was that, at pH 7.3 (which was required for the action of the phosphatase) and in presence of 25 mM $MgCl_2$, a substantial amount of the ADP-glucose (17%) was degraded chemically during the dephosphorylation step.[9b] It was known that alternatively ADP-glucose could be separated from AMP and ADP by anion-exchange chromatography (AEC). However, in strong contrast to our previous study in absence of polyP,[26] all compounds co-eluted from a 1 mL SuperQ-650M column, despite the use of shallow gradient in sodium acetate for optimum separation (Figure 6A). Therefore, this indicated the need to remove polyP from the reaction mixture in order to take advantage of the established methods for NDP-sugar isolation.

Employing fractional precipitation with ethanol at 4°C (Figure S12), it was possible to separate polyP as a glassy solid from ADP-glucose and residual AMP and ADP. The polyP precipitated on adding between 0.5 and 1.5 sample volumes of ethanol while the product and the adenosine phosphates remained fully soluble under these conditions. The beneficial effect of having removed the polyP on AEC efficiency in separating ADP-glucose from AMP and ADP is shown in Figure 6B. Despite use of a 20-fold increased sample loading compared to Figure 6A, all compounds were baseline separated by the same AEC protocol, so enabling convenient isolation of the ADP-glucose. It is worth emphasizing that the simple,

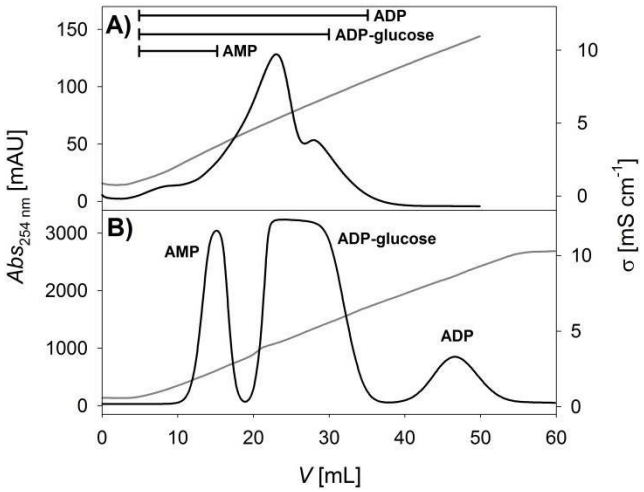


Figure 6. Separation of ADP-glucose from AMP and ADP by AEC before (A) and after (B) removal of polyP is shown. A gradient from 20 to 510 mM sodium acetate, pH 4.3, over 50 column volumes was used. In the absence of polyP (B), this gradient was followed by 10 min of an isocratic flow at 510 mM sodium acetate. UV absorbance at 254 nm (black), conductivity (grey).

yet efficient strategy of removing polyP by precipitation with ethanol could be applicable generally in the isolation of NDP-sugars obtained in syntheses involving PPK-catalyzed phosphorylations.

Using the protocol just established, ADP-glucose was isolated at preparative scale from a 10 mL sample (after polyP removal) containing 334 mg product. Table S1 summarizes the results and Figure S13 shows performance of the AEC for a 40 mL column. Sodium acetate in the product was removed by ethanol precipitation and ADP-glucose (155 mg) was obtained as a white powder by freeze drying from solution in water. The only moderate yield (48%) is due to ~10% product loss in each step, primarily as a (well-known) consequence of the relatively small scale used. Employing a similar procedure for the isolation of UDP-glucose at gram scale, lacking the precipitation of polyP however, we obtained a product recovery of 81%. It should be recognized therefore that the main interest here was not maximizing the isolated yield but demonstrating that the overall process is feasible for the production of highly pure ADP-glucose.

Analysis of the final product by HPLC, ^{1}H- and ^{13}C-NMR (Figure S14 – S16) confirmed its identity and revealed an excellent ADP-glucose purity with a mass percentage of 98.5% or greater. Around 1% ethanol remained as main impurity and additionally small amounts of AMP (≤ 0.25%), ADP (≤ 0.1%) and acetate (≤ 0.1%) were detected. Therefore, these results show that the PPK-SuSy cascade reaction combined with the suitable downstream procedure offers straightforward synthesis of highly pure ADP-glucose from inexpensive substrates. Other NDP-glucoses (Figure 3) could be prepared similarly.

1.3. Conclusion

In this study we demonstrate that PPKs allow straightforward conversion of NMP to provide NDP, which serves as high value precursor for NDP-sugar synthesis by reverse glycosylation. For efficient synthesis of NDP-glucose from inexpensive NMP, polyP and sucrose by PPK-SuSy cascade reaction, the interplay of all process steps required careful attention. In particular the use of polyP had a large impact on product isolation. Although NDP-sugar purification in presence of polyP by AEC was previously reported,[14a] we demonstrated that the efficiency of AEC is dramatically improved when polyP is removed by ethanol precipitation. In depth evaluation of ADP-glucose production demonstrated that the PPK-SuSy process is well competitive with alternative strategies which either rely on expensive precursors or require a multitude of enzymatic steps. Conversion yields of up to 81% and outstanding final product concentrations of up to 81 mM (48 g L^{-1}) were combined with an excellent purity of the isolated product (≥ 98.5%). However, we also recognized that metal ion chelation by phosphate groups triggered precipitation. To suppress this effect we suggest gradual addition of polyP in fed-batch conversions. By avoiding substrate inhibition the rate limiting PPK module should also kinetically profit when polyP is maintained at a low level throughout the conversion. Herein studied *Aj*PPK and *Mr*PPK displayed a broad acceptor spectrum but formation of purine containing ADP and GDP was clearly preferred. Therefore synthesis of pyrimidine based UDP-, CDP- and dTDP-glucose could not match the efficiency of ADP-glucose production. Although PPKs typically prefer purines also a bias towards pyrimidine containing nucleobases was recognized.[11, 16] Identification of such enzymes could further boost the application of the PPK-SuSy cascade reaction for straightforward synthesis of NDP-glucose. Because nucleotide phosphorylation is key for cost-efficient production of all NDP-sugars, the herein described synthetic application of a PPK is of general importance in the ongoing effort to overcome the current donor shortage which restricts synthetic application of GTs.

1.4. Experimental Section

1.4.1. Materials

PolyP (115% H_3PO_4 basis) was obtained from Sigma-Aldrich (Vienna, Austria) and nucleotides were purchased from Carbosynth (Berkshire, UK). CIAP was from New England Biolabs (Ipswich, MA, US) and SuperQ-650M was from Tosoh Bioscience (Tokyo, Japan).

1.4.2. Enzyme preparation

Recombinant expression of *Gm*SuSy (GenBank: AAC39323.1) and *Ac*SuSy (GenBank: AIA55343.1) in *E. coli* and their purification by Strep- and His-tag affinity chromatography were described elsewhere in detail.[18a, 19]

The pET-28a vectors for expression of *Aj*PPK (GenBank: BAC76403.1) and *Mr*PPK (GenBank: AGK05310.1) were received from Prof. Jennifer Andexer (Institute of Pharmaceutical Sciences, University of Freiburg, Germany). Codon optimized genes were inserted into *Nde*I and *Hind*III sites for expression as fusion proteins with N-terminal His-tag. Expression strains were created by transformation of *E. coli* BL21-Gold (DE3) cells. The strains were cultivated in 1 L baffled shake flask containing 300 mL lysogeny broth medium supplemented with 50 µg mL^{-1} kanamycin on a rotary shaker at 37°C and 120 rpm. When the optical density at 600 nm reached 0.8 - 1.0 enzyme expression was induced by addition of 500 µM isopropyl β-D-1-thiogalactopyranoside (IPTG). After overnight expression at 18°C, cells were harvested by centrifugation (5000 rpm, 30 min, 4°C). Cell extracts were prepared by French press as described elsewhere.[18a] For PPK purification two 5 mL His Trap FF columns (GE Healthcare; Vienna, Austria) were connected in series on an ÄktaPrime plus system (GE Healthcare). A constant flow rate of 2 mL min^{-1} was applied. A 50 mM Tris buffer (pH 7.4) containing 500 mM NaCl and 20 mM imidazole was used to equilibrate the columns and to wash off unbound proteins. Then PPKs were eluted in a single peak during a 100 mL long gradient from 20 to 250 mM imidazole.

Using centrifugal concentrators, enzymes were buffer exchanged to 25 mM HEPES, pH 7.0. *Ac*SuSy, *Mr*PPK and *Aj*PPK were stored at 4°C and *Gm*SuSy was kept at -20°C. Protein concentrations were determined photometrically at 280 nm and protein purities were assessed by SDS-PAGE.

1.4.3. HPLC-based activity assay

NMP, NDP, NTP and NDP-glucose were quantified by reversed-phase ion-pairing HPLC. Typically 5 µL of solutions containing an overall nucleoside concentration of 1 mM were loaded on a Kinetex™ C18 column (5 µm, 100 Å, 50 × 4.6 mm). Compounds with the same nucleobase were baseline separated by 6.5 min long isocratic runs using 20 mM phosphate buffer, pH 5.9 containing 40 mM tetra-*n*-butylammonium bromid (TBAB) and 12.5 % acetonitrile. The flow rate was 2 mL min^{-1} and the temperature was controlled to 35°C. Depending on the nucleobase quantifications were made by UV-detection at 253 (guanine), 259 (adenine), 262 (uracil), 267 (thymine) or 271 nm (cytosine).

To follow enzymatic conversions aliquots were withdrawn from reaction mixtures and enzymatic conversions were stopped by adding an equal volume of acetonitrile. Precipitated proteins were removed by centrifugation (13,200 rpm, 10 min) and appropriately diluted supernatant was applied to HPLC analysis.

1.4.4. Kinetic characterization of PPKs

All conversions contained 0.1 mg mL^{-1} BSA and were performed in 1.5 mL reaction tubes under agitation at 450 rpm. The pH of polyP stock solutions was set to the pH of the respective conversion and the pH of reaction mixtures was controlled before starting conversions by enzyme addition. Note that polyP concentrations correspond to the total phosphate residues not the number of polyP chains.

To test the acceptor spectrum of *Aj*PPK and *Mr*PPK 1 mM AMP, GMP, CMP, UMP or dTMP were phosphorylated. Reaction mixtures contained 6.6 mM polyP, 10 mM $MgCl_2$, 50 mM Tris, pH 7.2 and 50 µg mL^{-1} PPK. Conversions at 30°C were stopped after 6 h. The influence of the polyP concentration was evaluated in the range of 6.6 - 660 mM in reactions containing 1 mM AMP, 10 mM $MgCl_2$, 50 mM Tris, pH 7.1 and 50 µg mL^{-1} PPK. Conversions at 30°C were stopped after 6h. Phosphorylation of 1 mM AMP by 10 µg mL^{-1} *Mr*PPK was studied at 45°C in the pH range of 4.0 - 8.5. Reactions contained 10 mM $MnCl_2$ and were buffered by 50 mM Tris and 33 mM polyP.

1.4.5. Establishing PPK-SuSy cascade reactions

Reactions were performed as described above. 10 mM AMP was converted with *Gm*SuSy and *Ac*SuSy at 30 and

45°C, respectively. Reaction mixtures contained 33 mM polyP, 250 mM sucrose, 10 mM $MgCl_2$, 50 mM MES, pH 5.5, 50 µg mL^{-1} SuSy and 200 µg mL^{-1} *Mr*PPK. ADP-glucose formation was followed for 24 h. Under identical conditions conversion of 10 mM AMP, GMP, CMP, UMP and dTMP was tested with 300 µg mL^{-1} *Mr*PPK and 50 µg mL^{-1} *Ac*SuSy.

To optimize conversion of 100 mM AMP we varied the concentration of $MgCl_2$ and polyP as well as the pH. All reactions were performed at 45°C and contained 1 M sucrose, 50 mM MES, 50 µg mL^{-1} *Ac*SuSy and 100 µg mL^{-1} *Mr*PPK. $MgCl_2$ was varied from 10 to 100 mM in 48 h long conversions with 132 mM polyP at pH 5.5. Reactions with 132 - 265 mM polyP and 25 mM $MgCl_2$ were stopped after 24 h at pH 5.5. Finally pH was varied from 5.0 to 7.0 in 24 h long conversions with 132 mM polyP and 25 mM $MgCl_2$. Equation 1 was used to approximate the equilibrium constant (K_{eq}) of the SuSy reaction, whereby the concentrations of ADP and ADP-glucose were directly measured and those of fructose and sucrose were inferred from ADP-glucose formation and reaction stoichiometry.

$$K_{\mathrm{eq}} = \frac{c_{\mathrm{ADP-glucose}} \cdot c_{\mathrm{fructose}}}{c_{\mathrm{ADP}} \cdot c_{\mathrm{sucrose}}} \quad (1)$$

A reaction under optimized conditions (25 mM $MgCl_2$, 132 mM polyP and pH 5.5) was followed for 24 h.

1.4.6. Upscaled synthesis of ADP-glucose

To evaluate isolation of ADP-glucose the optimized conversion was upscaled from 0.5 to 150 mL. For temperature control to 45°C synthesis was performed in a double walled glass reactor with a diameter of 6.5 cm and a height of 6.4 cm. Throughout the 30 h long conversion agitation was applied by a magnetic stirrer (65 rpm). The pH was monitored and if necessary adjusted to 5.5 by addition of HCl. The reaction was terminated by removing enzymes through ultrafiltration by Vivaspin concentrators (10 kDa cut-off, Sartorius, Germany). The obtained mixture containing 59 mM ADP-glucose, 20 mM AMP, 6 mM ADP and polyP was used to isolate ADP-glucose.

1.4.7. Establishing isolation of ADP-glucose

To cleave phosphomonoesters 10 U mL^{-1} CIAP were added to the reaction mixture after setting the pH to 7.3. Dephosphorylation at 30°C was monitored over 24 h by HPLC analysis.

AEC was optimized with self-packed 1 mL SuperQ-650M columns at room temperature. Samples were loaded and separated with flow rates of 0.2 and 1 mL min^{-1}, respectively. After unbound compounds were washed off with 20 mM sodium acetate (pH 4.3); a 50 column volumes (CV) long gradient to 510 mM sodium acetate was applied for separation of AMP, ADP-glucose and ADP.

All precipitations involved overnight incubation at the specified temperature and ethanol content and separation of precipitate and soluble fraction was achieved by 10 min of centrifugation at 5,000 rpm and 4°C. To find conditions for selective polyP precipitation, the enzyme free reaction solution was incubated overnight at 4 and -20°C in presence of 0.5 to 2.5 equivalents of ethanol. The amounts of soluble and insoluble AMP, ADP-glucose and ADP were determined by HPLC.

1.4.8. Preparative isolation of ADP-glucose

Initially polyP was precipitated with 1.5 volumes of ethanol at 4°C. Before AEC dissolved nucleosides were precipitated at -20°C in presence of 5 equivalents of ethanol and redissolved in 10 mL water. AEC was performed as described above but on a 40 mL column with flow rates of 1 and 10 mL during sample loading and compound separation, respectively. ADP-glucose containing fractions were pooled and concentrated to 20 mL. To remove acetate, ADP-glucose was precipitated with 4 volumes of ethanol at -20°C. Dissolving of the insoluble fraction in 5 mL water was followed by a final precipitation of ADP-glucose with 5 volumes of ethanol at -20°C. The precipitate was dissolved in 1 mL of water and after freeze drying ADP-glucose was obtained as white powder. It was analysed by HPLC and NMR spectroscopy using a Varian Unity Inova 500 MHz spectrometer. The results of ^{1}H and ^{13}C NMR were in agreement with literature.[22b, 27] ^{1}H NMR (500 MHz, D_2O): δ=8.53 (s, 1H), 8.26 (s, 1H), 6.18 (d, *J* = 5.7 Hz, 1H), 5.65 (dd, *J* = 7.3, 3.5 Hz, 1H), ~4.8 (overlapping with H_2O signal), 4.60 (t, *J* = 4.4 Hz, 1H), 4.46 (q, *J* = 3.0 Hz, 1H), 4.30 (dd, *J* = 5.3, 3.1 Hz, 2H), 3.99 – 3.76 (m, 4H), 3.58 (dt, *J* = 9.8, 3.2 Hz, 1H), 3.50 (t, *J* = 9.7 Hz, 1H); ^{13}C NMR (126 MHz, D_2O): δ=156.18, 153.48, 149.68, 140.41, 119.22, 96.22, 87.55, 84.47, 74.95, 73.50, 72.32, 72.25, 71.02, 69.90, 65.92, 61.03.

1.5. Acknowledgements

Financial Support from the EU FP7 project SuSy (Sucrose Synthase as Cost-Effective Mediator of Glycosylation Reactions) is gratefully acknowledged. Prof. Jennifer Andexer (Institute of Pharmaceutical Sciences, University of Freiburg, Germany) kindly provided the plasmids for expression of AjPPK and MrPPK.

1.6. References

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2. SUPPORTING INFORMATION

Biocatalytic Cascade of Polyphosphate Kinase and Sucrose Synthase for Synthesis of Nucleotide-activated Derivatives of Glucose

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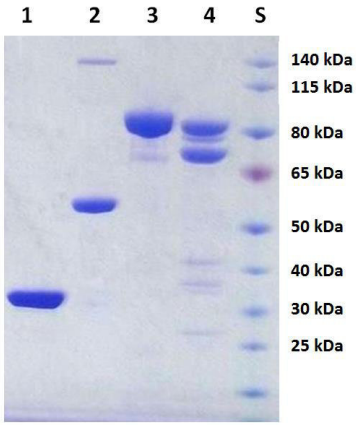


Figure S1. SDS-PAGE of *Mr*PPK, *Aj*PPK, *Gm*SuSy and *Ac*SuSy from *E. coli* overexpression cultures. *Gm*SuSy was purified by *Strep*-tag affinity chromatography while all other enzymes were purified by His-tag affinity chromatography. 1: *Mr*PPK (monomeric molecular weight: 31.6 kDa); 2: *Aj*PPK (monomeric molecular weight: 55.8 kDa); 3: *Gm*SuSy (monomeric molecular weight: 94.1 kDa); 4: *Ac*SuSy (monomeric molecular weight: 92.0 kDa); 4: S: PageRuler^{TM} Prestained Protein Ladder (Thermo Scientific).

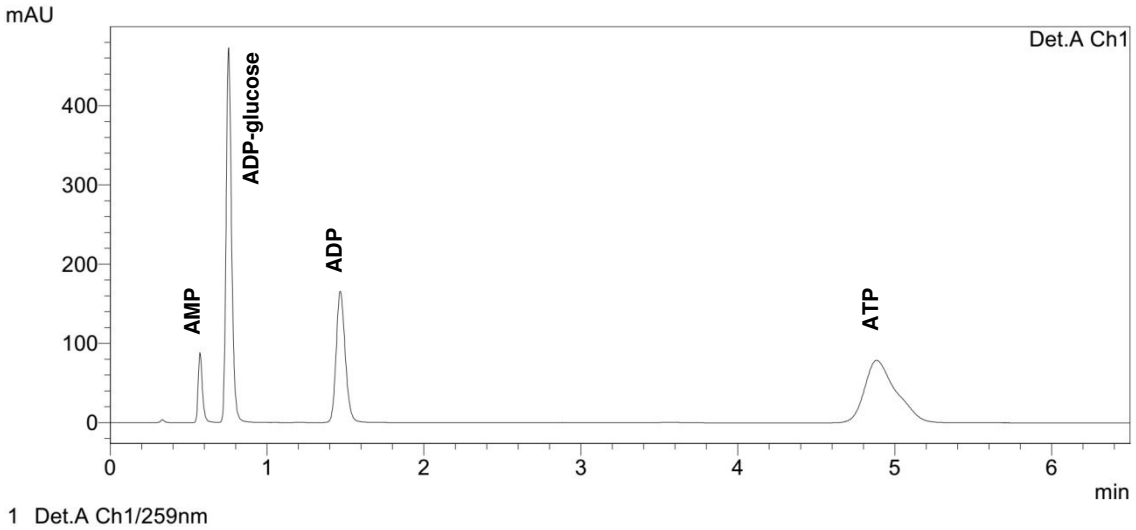


Figure S2. AMP, ADP-glucose ADP and ATP were quantified by reversed-phase C-18 HPLC using a TBAB based ion-pairing protocol with UV-detection at 259 nm.

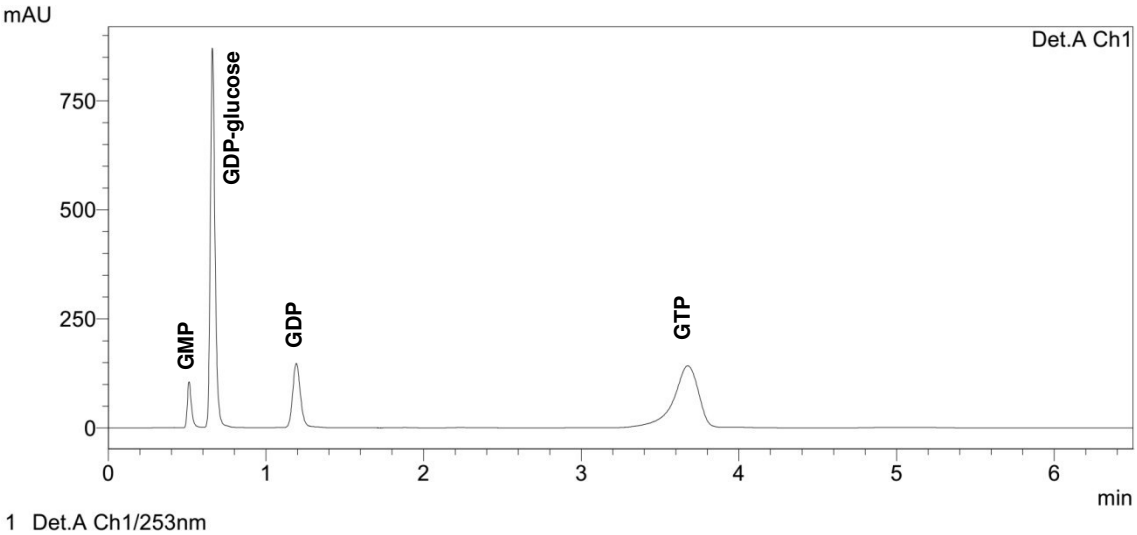


Figure S3. GMP, GDP-glucose GDP and GTP were quantified by reversed-phase C-18 HPLC using a TBAB based ion-pairing protocol with UV-detection at 253 nm.

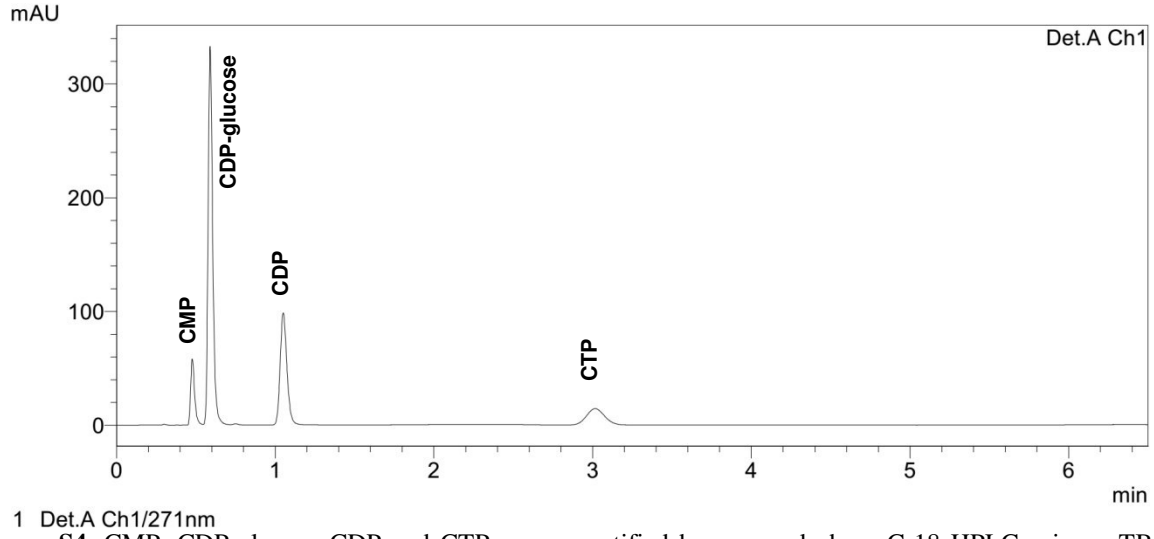


Figure S4. CMP, CDP-glucose CDP and CTP were quantified by reversed-phase C-18 HPLC using a TBAB based ion-pairing protocol with UV-detection at 271 nm.

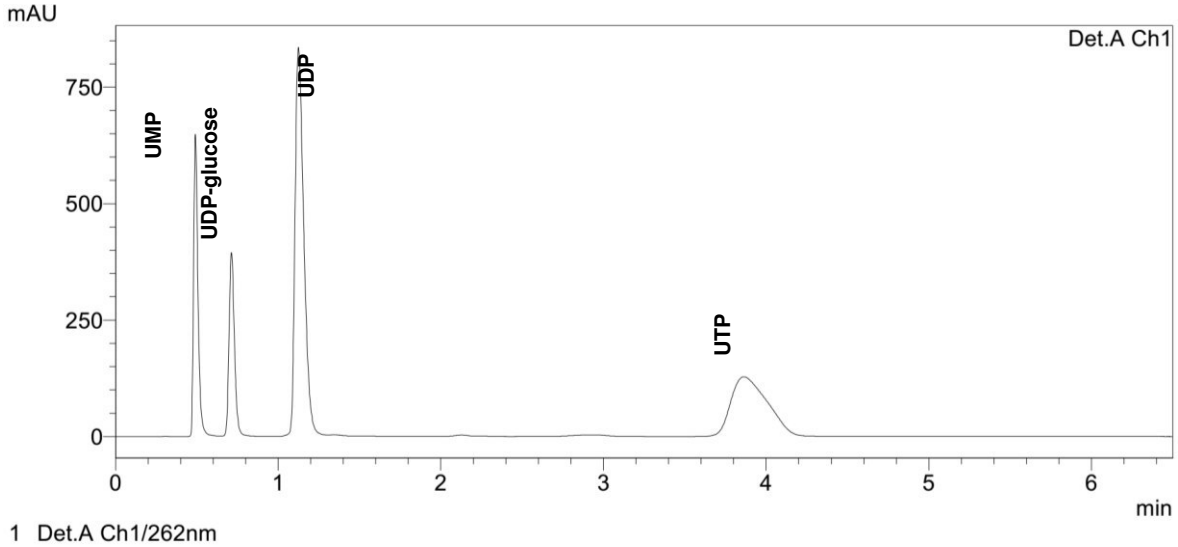


Figure S5. UMP, UDP-glucose UDP and UTP were quantified by reversed-phase C-18 HPLC using a TBAB based ion-pairing protocol with UV-detection at 262 nm.

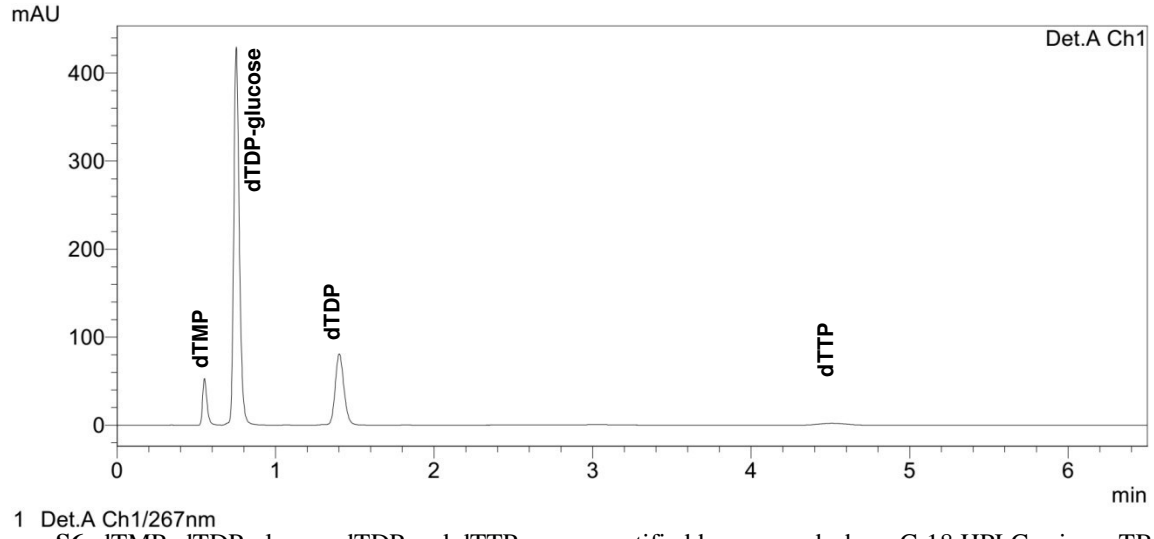


Figure S6. dTMP, dTDP-glucose dTDP and dTTP were quantified by reversed-phase C-18 HPLC using a TBAB based ion-pairing protocol with UV-detection at 267 nm.

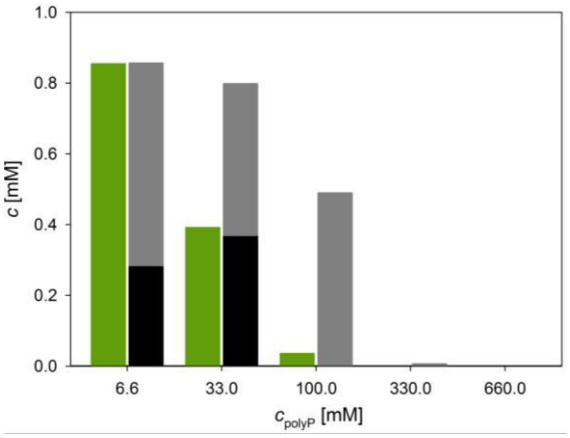


Figure S7. The negative effect of high polyP concentrations on AMP conversion was much more pronounced with *Aj*PPK than with *Mr*PPK. *Aj*PPK formed exclusively ADP (green) but *Mr*PPK partially converted ADP (grey) further to ATP (black). Reaction conditions: 1 mM AMP, 50 µg mL^{-1} PPK, pH 7.1, 6 h, 30°C.

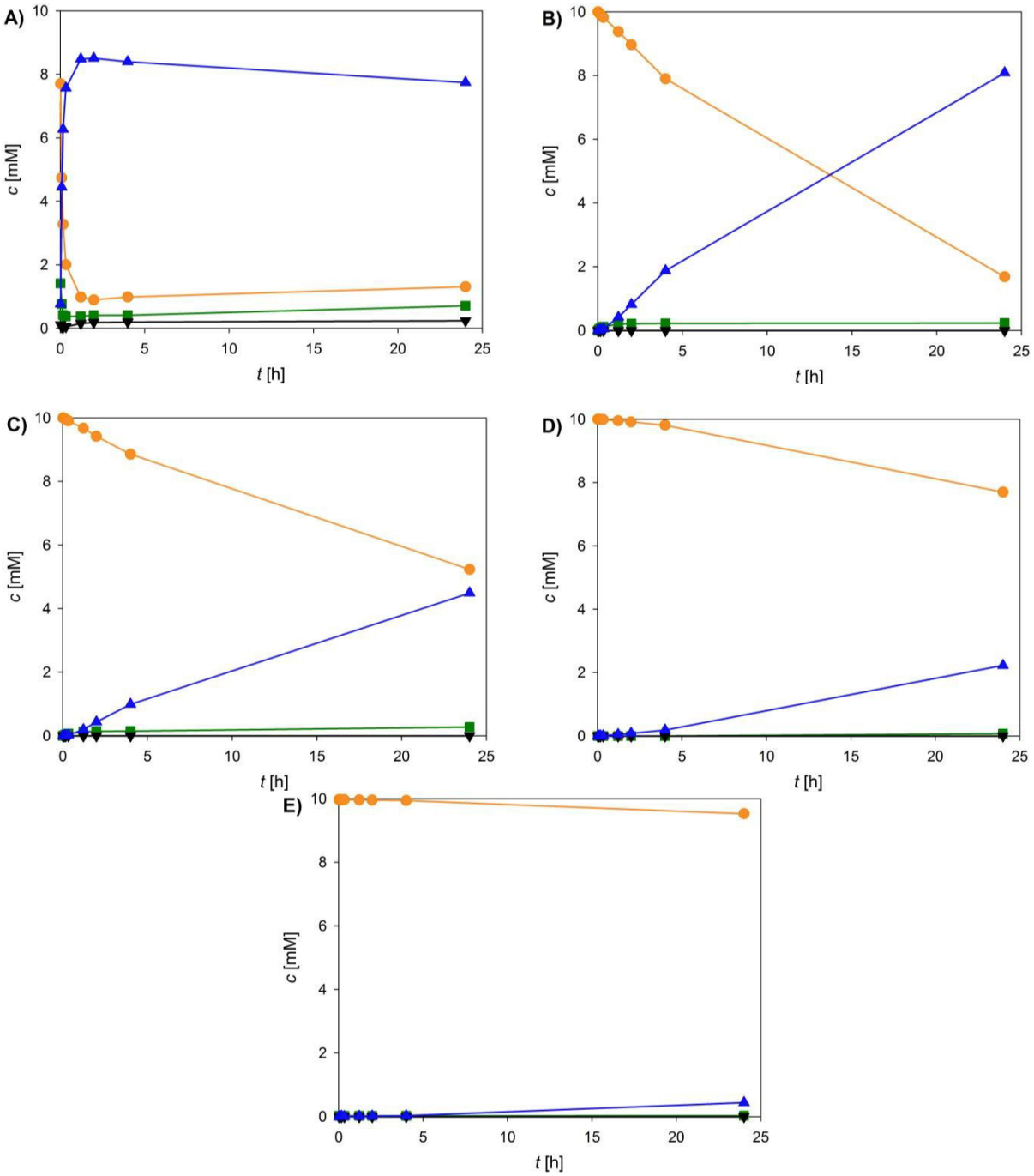


Figure S8. AMP (A), UMP (B), CMP (C), GMP (D) and dTMP (E) were used to synthesize NDP-glucose by the *Mr*PPK-*Ac*SuSy cascade reaction at 45°C. Reactions mixtures contained 10 mM NMP, 33 mM polyP, 250 mM sucrose, 300 µg mL^{-1} *Mr*PPK, 50 µg mL^{-1} *Ac*SuSy and 50 mM MES, pH 5.5.

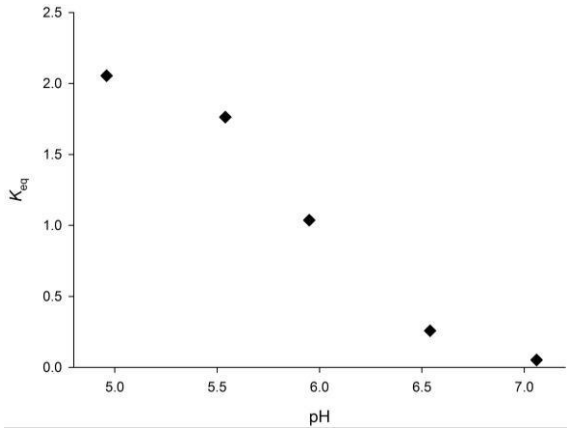


Figure S9. The equilibrium constant (K_{eq}) of ADP-glucose formation by SuSy showed a strong pH dependence. Final ADP and ADP-glucose concentrations from AMP conversions by *Mr*PPK-*Ac*SuSy cascade reactions were used to calculate K_{eq}. Data was extracted from Figure 4C. Reaction conditions: 100 mM AMP, 132 mM polyP, 1000 mM sucrose, 100 µg mL^{-1} *Mr*PPK, 50 µg mL^{-1} *Ac*SuSy, 45°C.

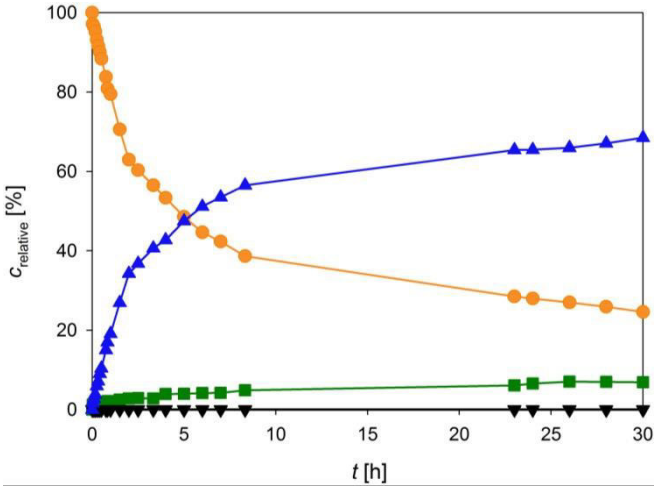


Figure S10. ADP-glucose synthesis by the *Mr*PPK-*Ac*SuSy cascade reaction was upscaled to a working volume of 150 mL (100 mM AMP, 1000 mM sucrose, 132 mM polyP, 25 mM $MgCl_2$, pH 5.5). The relative content of soluble AMP (orange), ADP (green), ADP-glucose (blue), ATP (black) is shown. Throughout the conversion 16% of adenosine containing compounds precipitated. The moderate final ADP-glucose yield of 61% (based on soluble AMP) is an indication that precipitation caused depletion of polyP.

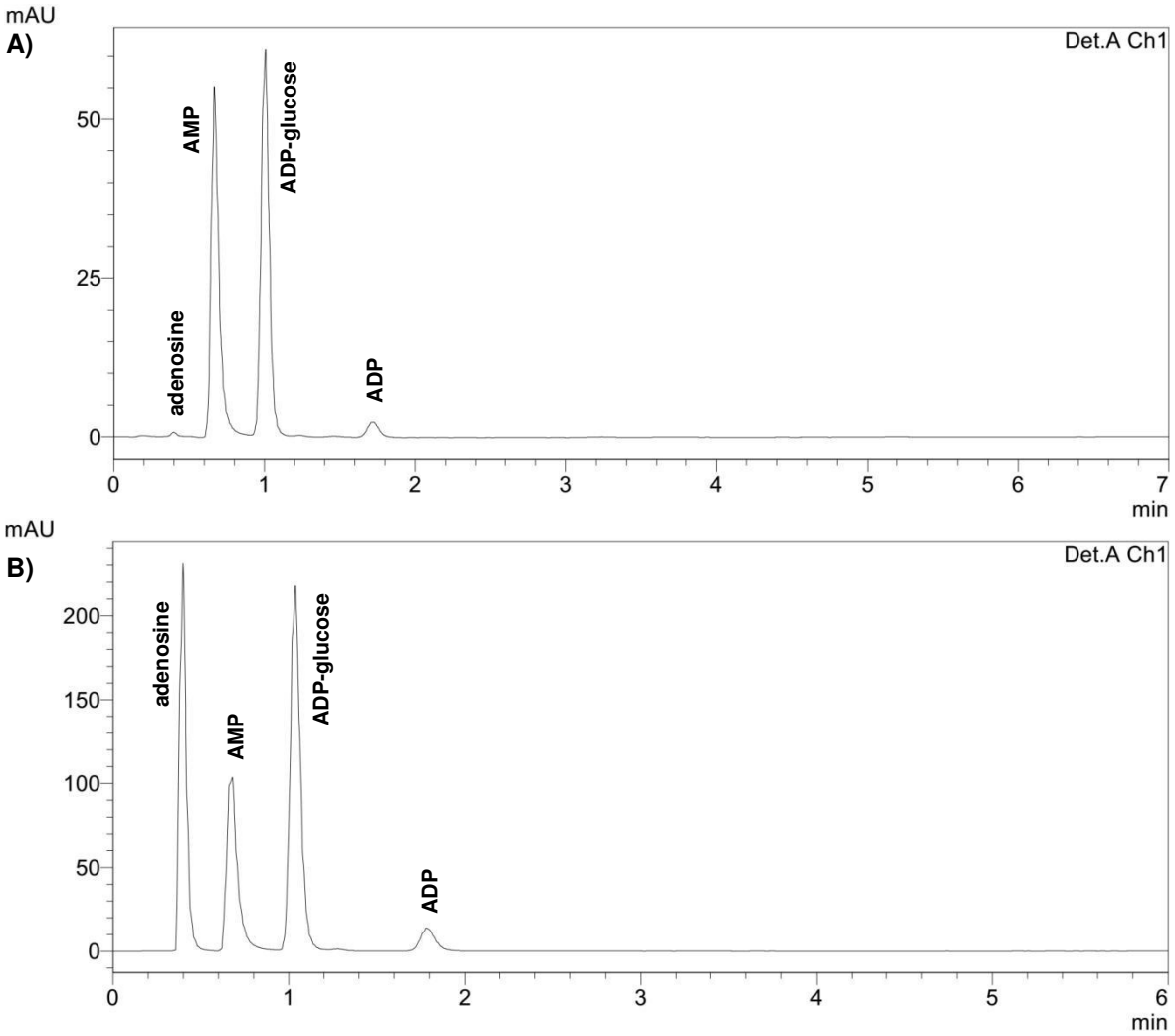


Figure S11. PolyP containing reaction mixtures from *Mr*PPK-*Ac*SuSy cascade reactions were treated by 10 U mL^{-1} CIAP at 30°C for 24 h. HPLC elution profiles before (A) and after (B) dephosphorylation show that AMP and ADP were only partially converted to adenosine. Simultaneously 17% of ADP-glucose were lost by decomposition to AMP and glucose-1,2-monophosphate.

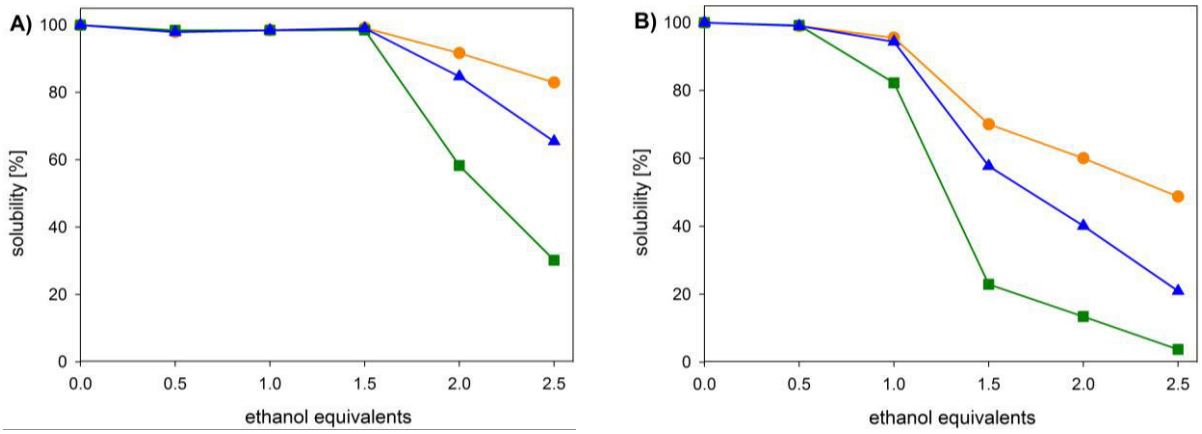


Figure S12. The solubility of ADP-glucose (blue), AMP (orange) and ADP (green) was tested in presence of various ethanol concentrations at A) 4 and B) -20°C. A protein free reaction mixture containing 57 mM ADP-glucose, 20 mM AMP, 6 mM ADP and polyP was used. Samples were incubated overnight and centrifuged to separate soluble fraction and precipitate, which were analyzed separately by HPLC.

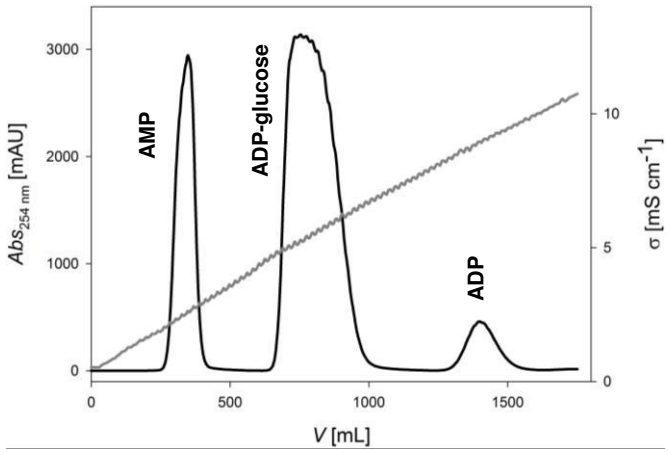


Figure S13. ADP-glucose was separated from AMP and ADP by preparative AEC using a 40 mL Toyopearl SuperQ-650M column. A 50 CV long gradient from 20 to 510 mM sodium acetate was applied at pH 4.3 using a flow rate of 10 mL min^{-1}. UV absorbance at 254 nm (black), conductivity (grey).

Table S1. Purification of ADP-glucose by AEC after fractionated polyP precipitation

step	T [°C]	ethanol equivalents	recovery [%] step	recovery [%] total	ADP-glucose content[a] [%]	$m_{ADP\text{-}glucose}$ [mg]
end of reaction					68.5	334
polyP precipitation	4	1.5	83.3	83.3	66.4	278
1st ADP-glucose precipitation	-20	5	93.1	77.6	70.7	259
AEC			81.9	63.5	99.7	212
2nd ADP-glucose precipitation	-20	4	84.4	53.6	99.7	179
3rd ADP-glucose precipitation	-20	5	95.5	51.2	99.7	171

[a] Based on nucleoside quantification by HPLC measurement.

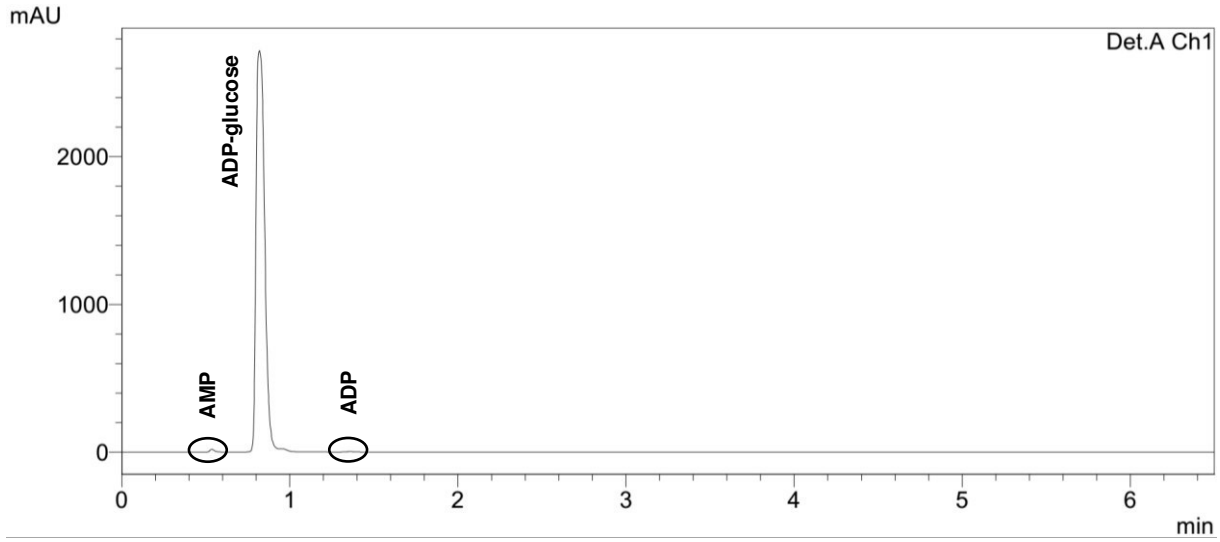


Figure S14. HPLC analysis of purified ADP-glucose revealed only ≤ 0.25% AMP and ≤ 0.10% ADP as remaining detectable impurities.

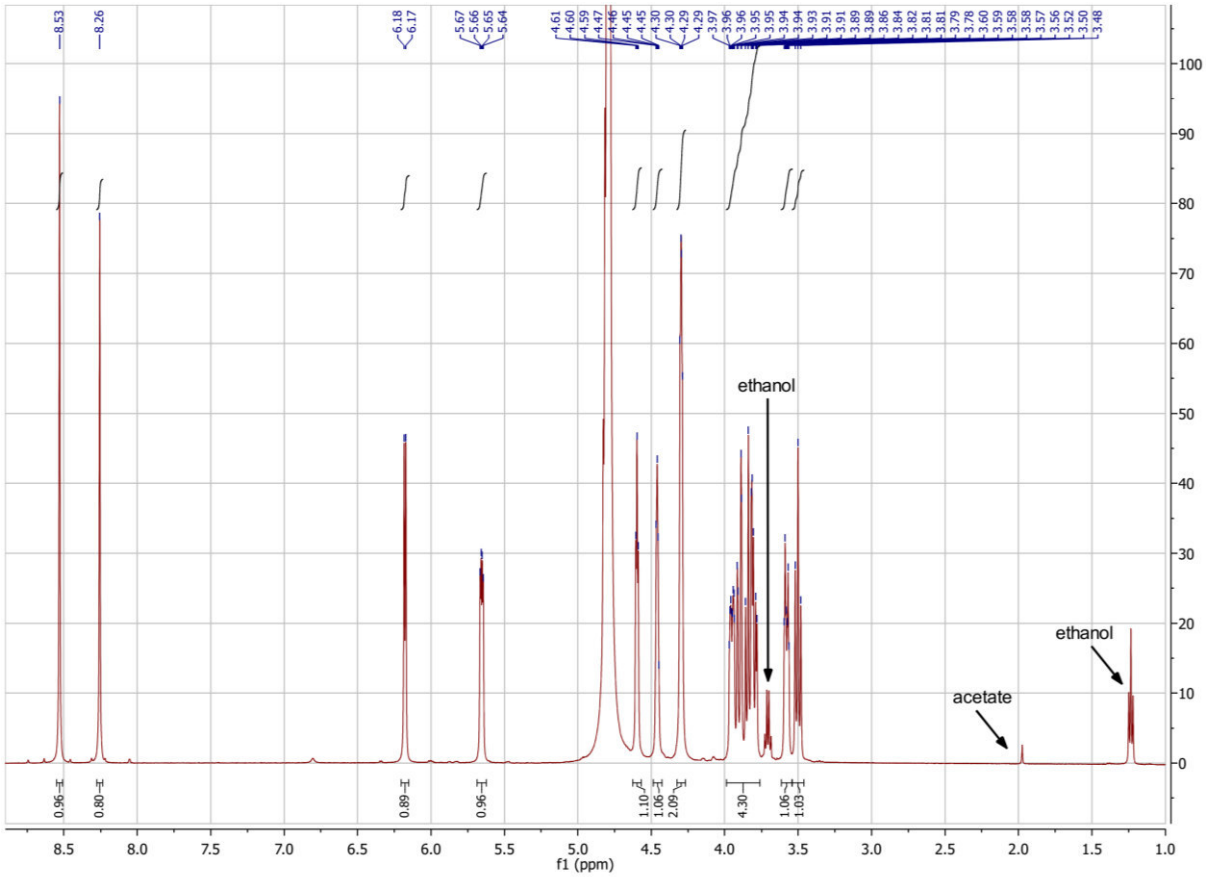


Figure S15. [1]H-NMR of purified ADP-glucose was consistent with literature reports. The main impurities were ethanol and acetate with mass percentages of around 1 and 0.1%, respectively.

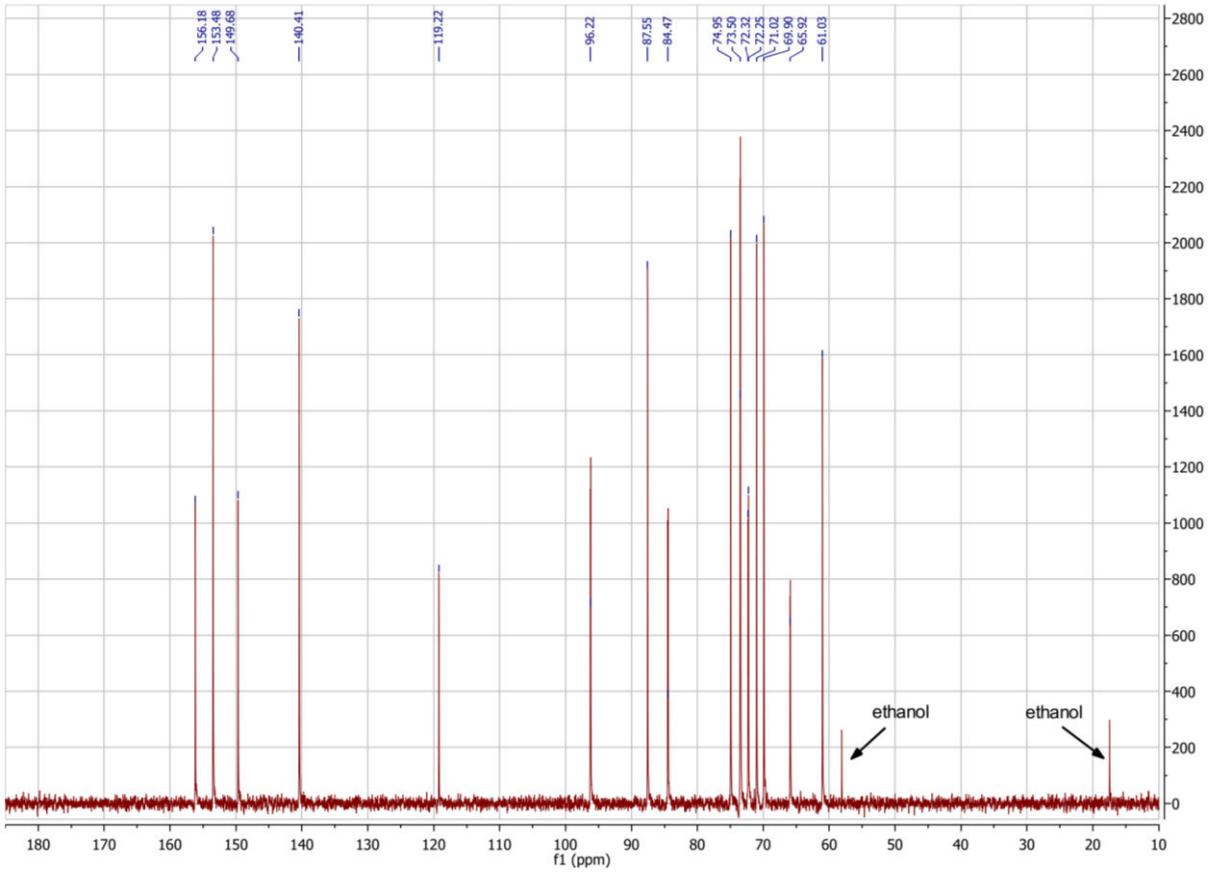


Figure S16. ^{13}C-NMR of purified ADP-glucose was consistent with literature reports. Ethanol was the only detectable impurity.

3. APPENDIX

Cloning, expression and initial conversions

Enzymes: IPP, phoA, UMPK, CMPK, GMPK, AMPK, NDK, *Ec*PPK, *Mr*PPK, *Sm*PPK, *Aj*PPK and *Ac*SuSy

3.1. Gene Overview

Information on all enzymes used within cloning work and protein expression is summarized in Table 1. Full enzyme names can be found in Table 2.

Table 1: Detailed gene information

enzyme	*E.C. Number*	*GenBank*	*MW [kDa]*	*Protein ID*	*size [bp]*
IPP	3.6.1.1	KP670718.1	43.7	AJH12852.1	540
UMPK	2.7.1.48	EMV48922.1	28.3	AJH09128.1	735
AMPK	2.7.4.3	AAB40228.1	30.3	AJH09378.1	654
phoA	3.1.3.1	M13345.1	47.1	AAA83893.1	1428
CMPK	2.7.4.14	KFH76471.1	24.7	AJH09806.1	693
*Ac*SuSy-short	2.1.4.13	AIA55343.1.	91.2	A0A059ZV61	2391
GMPK	2.7.4.8	AAN82909.1	21.7	AJH12276.1	633
NDK	2.7.4.6	EMV21356.1	17.6	AJH11206.1	441
*Ec*PPK	2.7.4.1	BAJ44283.1	82.6	AJH11189.1	2076
*AjP*PK	2.7.4.1	AB092983.1	55.8	BAC76403.1	1428
*Mr*PPK	2.7.4.1	AGK05310.1	31.6	n.a.	804
*Sm*PPK	2.7.4.1	HG799420.1	34.8	DL73027.1	903

Table 2: Abbreviations and full names of enzymes

enzyme abbr.	*full name*
IPP	Inorganic Pyrophosphatase
UMPK	Uridine Monophosphate Kinase
AMPK	Adenosine Monophosphate Kinase
phoA	Alkaline Phosphatase
CMPK	Cytidine Monophosphate Kinase
*Ac*SuSy-short	Sucrose Synthase from Acidithiobacillus caldus
GMPK	Guanosine Monophosphate Kinase
NDK	Nucleotide Diphosphate Kinase
*Ec*PPK	Polyphosphate Kinase from Escherichia coli
*AjP*PK	Polyphosphate Kinase from Acinetobacter jonsonii
*Mr*PPK	Polyphosphate Kinase from Meiothermus ruber
*Sm*PPK	Polyphosphate Kinase from Sinorhizobium meliloti

3.2. Construction of expression vectors

3.2.1. Restriction digestion method: UMPK, AMPK, CMPK, phoA, IPP and *Ac*SuSy-short

Genes listed in Table 3 were amplified from the *E. coli* genome and Phusion®DNA polymerase was applied in a standard PCR protocol (Table 4). The appropriate temperature gradient used for amplification is listed in Table 5. Sequence information of the respective primers are shown in Table 6. *Ac*SuSy however was amplified from a pPCXP34-*Ac*SuSy template provided by the group of Prof. Tom Desmet (Centre for Industrial Biotechnology and Biocatalysis, Ghent University, Belgium).

Table 3: Genes cloned with restriction enzymes

fragment	*fwd primer*	*rv primer*	*template*	*size [bp]*
IPP	IPP_*NdeI*_fw	IPP_*XhoI*_rv	*E. coli* genome	540
UMPK	UMPK_*NdeI*_fw	UMPK_*XhoI*_rv	*E. coli* genome	735
AMPK	AMPK_*NdeI*_fw	AMPK_*XhoI*_rv	*E. coli* genome	654
phoA	phoA_*Nde*I_fw	phoA_*XhoI*_rv	*E. coli* genome	1428
CMPK	CMPK_*Nde*I_fw	CMPK_*XhoI*_rv	*E. coli* genome	693
*Ac*SuSy-short	*Ac*SuSy-short_*NdeI*_fw	*Ac*SuSy_*XhoI*_rv	pCXP34_*Ac*SuSy	2391

Table 4: Components of a standard PCR mixture

solution	*V [µL]*
H_2O	28.7
5x Phusion High Fidelity Buffer	10
dNTP mix (2 mM each)	5
Gene template (diluted 1:5)	5
Primer fwd (10 pmol/µL)	2
Primer rev (10 pmol/µL)	2
Phusion®DNA Polymerase (2 U/µL)	0.3

Table 5: Temperature gradient for PCR protocol

step	*T [°C]*	*t [s]*	*cycles*
initial denaturation	98	45	1
denaturation	98	15	
annealing	57	20	35
extension	72	120	
final extension	72	420	1
soak	4	∞	1

Table 6: Primers used for IPP, UMPK, AMPK, CMPK and *Ac*SuSy-short amplification

name	*sequence*	*size [bp]*
IPP_*NdeI*_fw	catatgagcttactcaacgtccctgcgg	28
IPP_*XhoI*_rv	ctcgcgttatttattctttgcgcgctcgaagg	32
UMPK_*NdeI*_fw	catatggctaccaatgcaaaacccgtc	27
UMPK_*XhoI*_rv	ctcgagttattccgtgattaaagtcccttcttttttc	36
AMPK_*NdeI*_fw	catatgcgtatcattctgcttggcgctcc	29
AMPK_*XhoI*_rv	ctcgagttagccgaggatttttttccagatcagcg	34
CMPK_*NdeI*_fw	catatgacggcaattgccccggttattacc	30
CMPK_*XhoI*_rv	ctcgagttatgcgagagccaatttctggcgc	31
*Ac*SuSy-short_*NdeI*_fw	catatgattgaagccctgcgccaac	25
*Ac*SuSy_*XhoI*_rv	ctcgagttattccatcgggactgcgtgtgcc	31

All genes listed in Table 3 were cloned using a restriction digestion method. Thereby two flanking restriction sites (*Nde*I and *Xho*I) were introduced by PCR by addition of the respective primers given in Table 6. Genes were then cloned into a pJET vector via blunt-end cloning protocol (Thermo Scientific) prior to transformation into *E. coli* DH5 alpha cells. For protein expression in *E. coli* all genes, except *Ac*SuSy, were planned to be integrated in a pET-*Strep*3 *E. coli* expression vector.[1] PET-*Strep*3, which is a custom made derivate of pET-24d that enables protein expression with N-terminally fused *Strep*-tag II, was received from the

group of Prof. Robert Edwards (Centre for Bioactive Chemistry, Durham University, UK). *Ac*SuSy however was planned to be integrated in a pET-*Strep*3_TEV vector.
For construction of expression vectors a restriction digest of the isolated plasmids from pET-*Strep*3, pET-*Strep*3_TEV and the genes integrated into the pJET vector was made. After dephosphorylation (1 µL FastAP™ Thermosensitive Alkaline Phosphatase in a total volume of 50 µL) of the target vectors, purification via agarose gel electrophoresis and determination of the yielded concentrations, genes were ligated into the respective sites of pET-*Strep*3 or pET-*Strep*3_TEV for expression as N-terminal *Strep*-tagged fusion protein.
Ligation was performed with 1 µL T4 DNA Ligase and 2 µL 10x T4 DNA Ligase buffer in a total volume of 20 µL at 4°C overnight. Molar ratios of gene to vector were varied from 1:3 to 1:5 (vector : insert) for individual genes.

3.2.2. Circular Polymerase Extension Cloning: GMPK, NDK and *Ec*PPK

Genes listed in Table 7 were cloned using Circular Polymerase Extension Cloning (CPEC) as described by Quan et al (2009). [2] No restriction digestion, ligation, or single-stranded homologous recombination is required with this method. For amplification of the respective genes the *E. coli* genome was used as template and Phusion®DNA polymerase was applied in a standard PCR protocol (Table 4, Table 5) with primers listed in Table 7. CPEC-primers were used to create overlapping regions between vector and insert, which were amplified previously. Polymerase was then used to extend the overlapping regions to form a complete circular plasmid. Molar ratios of 5:1 (gene : vector) were used therefore. CPEC standard protocol and individual temperature gradients are listed in Table 8, Table 9 and Table 10.

Table 7: Primers used for GMPK, NDK and *Ec*PPK amplification and CPEC

name	***sequence***	***size [bp]***
GMPK_*NdeI*_fw	catatggctcaaggcacgctttatattg	28
GMPK_*XhoI*_rv	ctcgagtcagtctgccaacaatttgctgattaaag	35
CPEC-GMPK_fwd	gttcgagaaaggcttaattaaccatatggctcaaggcacgctttatattgtttc	54
CPEC-GMPK_rv	gtggtggtggtggtgctcgagtcagtctgccaacaatttgctgat	45
CPEC-GMPK-Strep_fwd	gaaacaatataaagcgtgccttgagccatatggttaattaattaagcctttctcgaac	59
CPEC-GMPK-Strep_rv	atcagcaaattgttggcagactgactcgagcaccaccaccacca	44
NDK_fwd	atggctattgaacgtacttttccatc	27
NDK_rv	ttaacgggtgcgcgggc	17
CPEC-NDK_fwd	gttcgagaaaggcttaattaaccatatggctattgaacgtacttttccatc	54
CPEC-NDK_rv	gtggtggtggtggtgctcgagttaacgggtgcgcgggc	38
CPEC-NDK-Strep_fwd	ggaaaaagtacgttcaatagccatatggttaattaagcctttctcgaac	49
CPEC-NDK-Strep_rv	gcccgcgcacccgttaactcgagcaccaccaccaccac	38
PPK_fwd	atgggtcaggaaaagctatacatcg	25
PPK_rv	ttattcaggttgttcgagtgatttgatgtag	31
CPEC-PPK_fwd	gttcgagaaaggcttaattaaccatatgggtcaggaaaagctatacatcg	50
CPEC-PPK_rv	gtggtggtggtggtgctcgagttattcaggttgttcgagtgatttgatgtag	52
CPEC-PPK-Strep_fwd	cgatgtatagcttttcctgacccatatggttaattaagcctttctcgaac	50
CPEC-PPK-Strep_rv	ctacatcaaatcactcgaacaacctgaataactcgagcaccaccaccaccac	52

Table 8: Components of a standard CPEC ligation mixture

solution	*V [µL]*
5x Phusion High Fidelity Buffer	10
dNTP mix (2 mM each)	2
DMSO	1.5
Phusion®DNA Polymerase (2 U/µL)	1
CPEC-insert CPEC-vector	ratio 5:1 (gene : vector)
H_2O	Up to 25 µL

Table 9: Temperature gradient for CPEC ligation of NDK

step	*T [°C]*	*t [s]*	*cycles*
initial denaturation	98	30	1
denaturation	98	10	8
ramp	0.1°C/sec from 70-55°C		
annealing	55	30	
extension	72	120	
final extension	72	300	1
soak	4	∞	1

Table 10: Temperature gradient for CPEC ligation of *Ec*PPK and GMPK

step	*T [°C]*	*t [s]*	*cycles*
initial denaturation	98	30	1
denaturation	98	10	8
annealing	55	30	
extension	72	180	
final extension	72	600	1
soak	4	∞	1

3.2.3. Polyphosphate Kinases

PPK2 genes from *Shinorizobium meliloti* (*Sm*PPK), *Meiothermus ruber* (*Mr*PPK) and *Acinetobacter johnsonii* (*Aj*PPK) were obtained from Prof. Jennifer Andexer (Institute of Pharmaceutical Sciences, University of Freiburg, Germany) in a pET28a-Vektor with an N-terminal His-tag. The vector sequence of pET28a can be found in paragraph 11.

3.3. Strain creation and enzyme expression

Obtained expression vectors were used for transformation of electro-competent DH5 alpha cells (self-prepared). 6 µL purified plasmid-DNA were desalted for 30 minutes and then mixed with 100 µL of cells and exposed to 1.8 kV for 4.8 msec and incubated with 600 µL prewarmed SOC-medium for 1 hour at 37°C. Transformed cells were selected on LB-kanamycin plates at 37°C. The correct sequence was verified by sequencing the entire genes (LGC Genomics, Berlin, Germany). The correct plasmids were then transformed into electro-competent *E. coli* BL21-Gold cells (self-prepared).
The recombinant cells for UMPK-Strep, AMPK-Strep, CMPK-Strep, GMPK-Strep, NDK-Strep, *Ec*PPK-Strep, *Sm*PPK-His, *Mr*PPK-His and *Aj*PPK-His expression were cultivated in baffled shaking flasks. Pre-cultures of 70 mL LB-medium containing 50 µg mL^{-1} kanamycin were inoculated with cells from LB-kanamycin plates. The medium was incubated at 37°C and 120 rpm overnight reaching an optical density at 600 nm of around 4. Main cultures of 300

mL LB-kanamycin medium were then inoculated to OD_{600} of 0.08 to 0.1 with the pre-culture and incubated at 37°C. As the main culture reached an optical density at 600 nm of 0.8 – 1.2 protein expression was induced with 150 µL of 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.5 mM final concentration) and incubated at 18°C overnight. The cells were harvested by centrifugation (30 min, 4°C, 5000 rpm) and resuspended in 5 mL water and aliquots were stored at -70°C.

3.4. Enzyme purification

Cells were disintegrated by repeated passage through a cooled French Pressure cell press (100 bar). Cell debris was removed by centrifugation at maximum rpm (13,200) at 4°C for 40 min. The supernatant was filtered through a 1.2 µm cellulose-acetate syringe filter before loading on purification columns.

3.4.1. Strep-tag purification

The supernatant of Strep-tagged enzymes was diluted twofold with washing buffer (100 mM Tris/HCl pH 8.0, 150 mM NaCl, 1 mM EDTA) to achieve a proper pH-value for purification (pH should be above 7.5 for best binding). Purification of enzymes (GMPK, NDK, UMPK, AMPK, CMPK, *Ec*PPK) was performed by Strep-tag affinity chromatography on 3 mL gravity flow columns as recommended by the manufacturer (IBA BioTAGnology, Göttingen, Germany).

3.4.2. His-tag purification

Purification of His-tagged enzymes was performed with a 5 mL HiTrap™ Chelating FF column as recommended by the manufacturer (GE-Healthcare) on an Äkta system. A constant flow of 2 mL min^{-1} was applied and 50 mM Tris buffer (pH7.4) containing 500 mM NaCl and 20 mM imidazole was used to equilibrate the columns and to wash off unbound proteins. Enzymes were eluted in a single peak during an appropriate gradient (50-100 mL long) from 20 to250 mM imidazole.

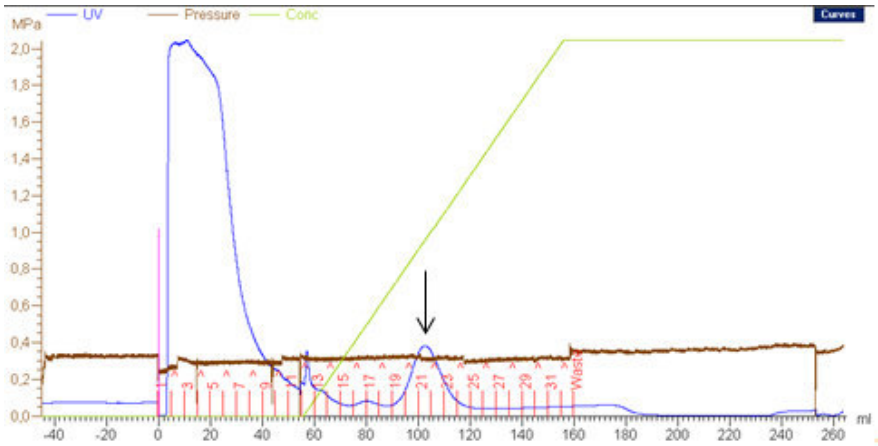


Figure 1: Purification of *Aj*PPK on an Äkta system

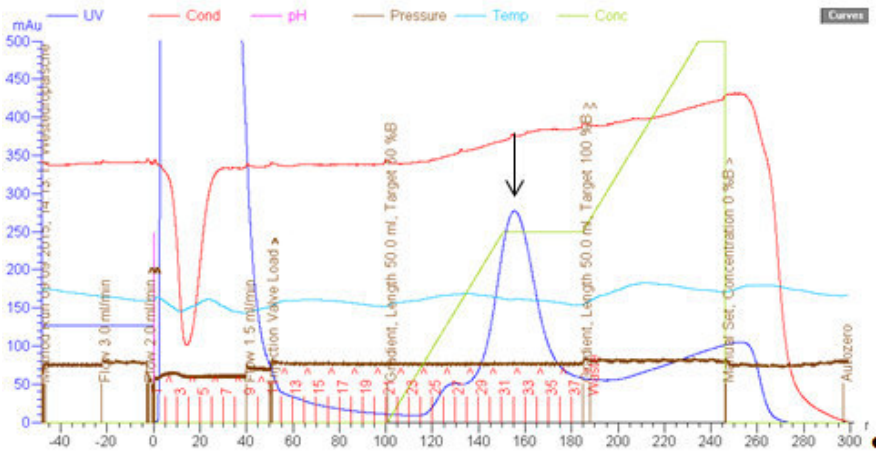


Figure 2: Purification of *Sm*PPK on an Äkta system

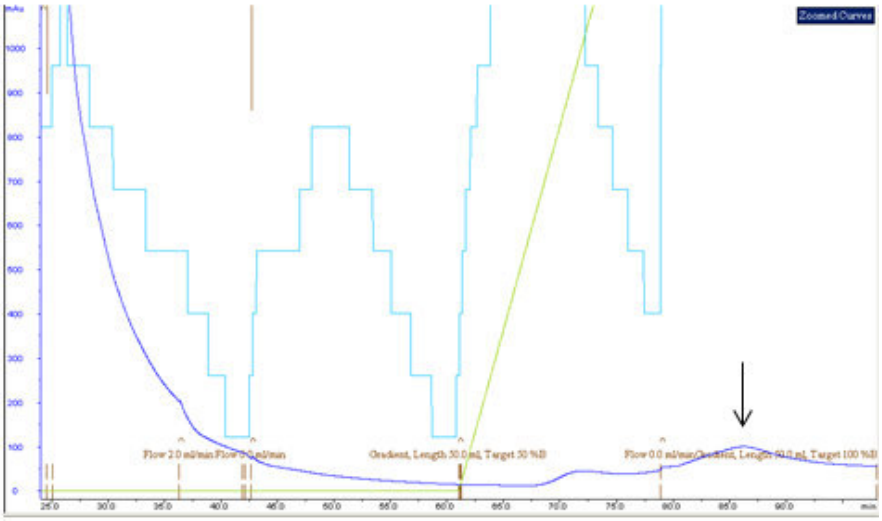


Figure 3: Purification of *Mr*PPK on an Äkta system

3.4.3. SDS PAGE analysis

To evaluate enzyme purity sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) was used. Different concentrations of the protein samples with 3 µL SDS dissociation buffer (NuPAGE® LDS Sample Buffer 4x) and water up to an end volume of 12 µL were prepared. The samples were denatured at 99°C for 10 min and then applied on the SDS gel (NuPage 4-12% Bis-Tris Gel). Protein fragments were separated within 60 min at 150 V. Coomassie brilliant blue dye (0.1% Coomassie R-250, 40% EtOH, 10% HAc) was used for staining the protein bands. Destaining was achieved by using a destain solution (30% EtOH, 10% HAc). PageRuler^{TM} Prestained Protein Ladder (Thermo Scientific) was used as molecular weight standard.

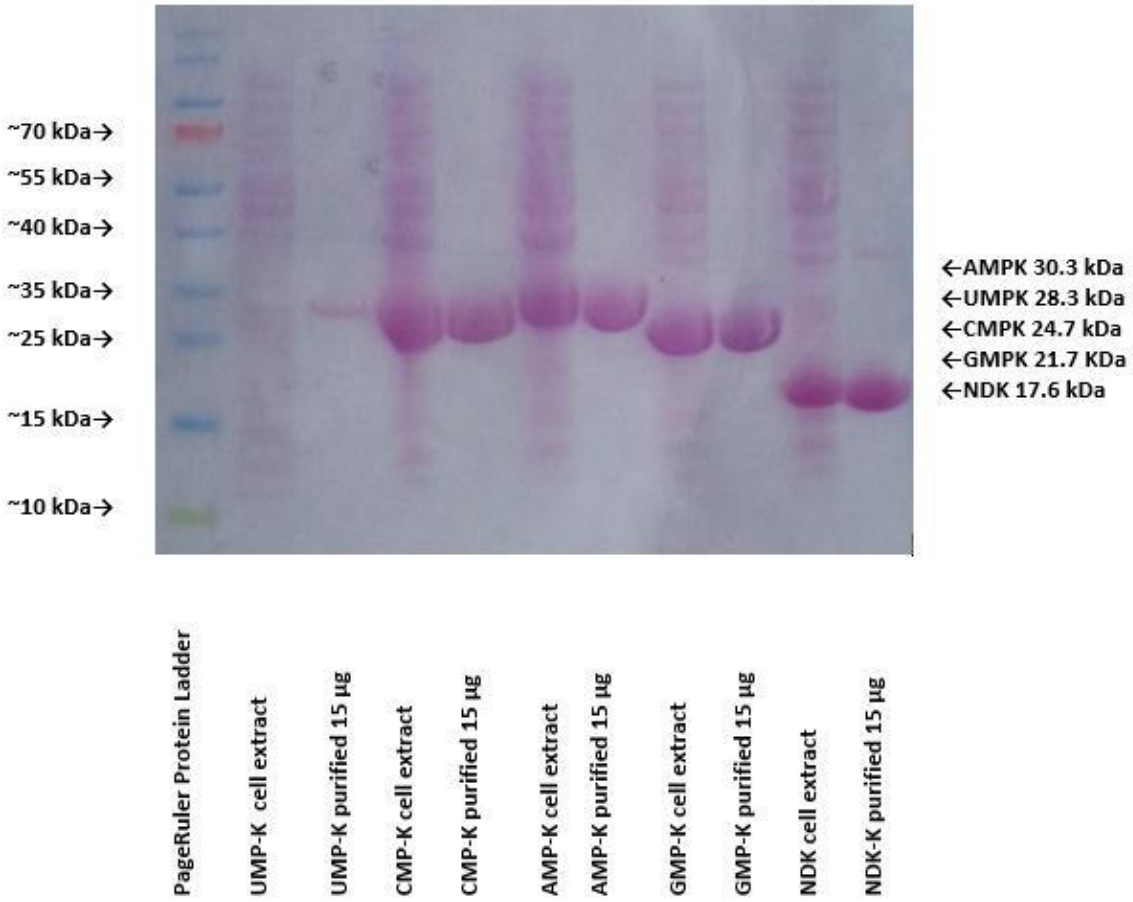


Figure 4: SDS-PAGE of UMPK CMPK, AMPK, GMPK and NDK purified by Strep-tag affinity chromatography

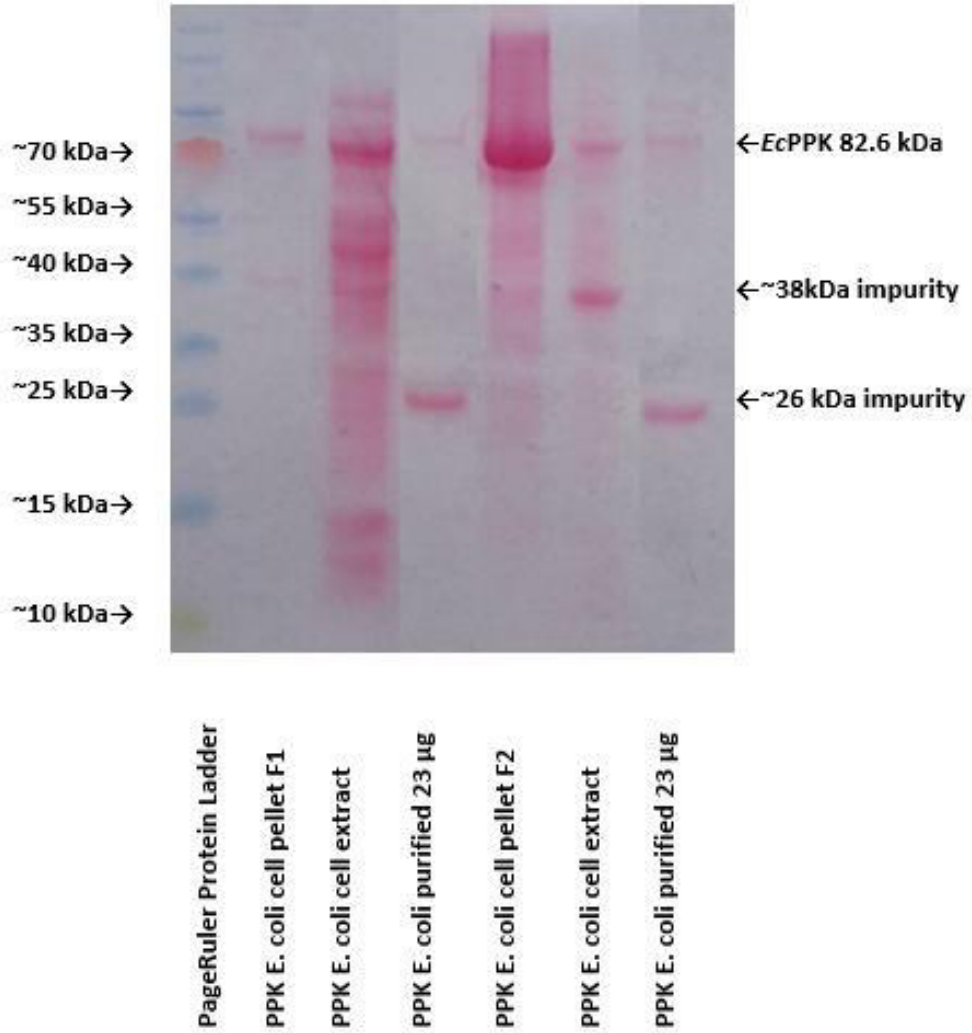


Figure 5: SDS-PAGE of *Ec*PPK purified by Strep-tag affinity chromatography

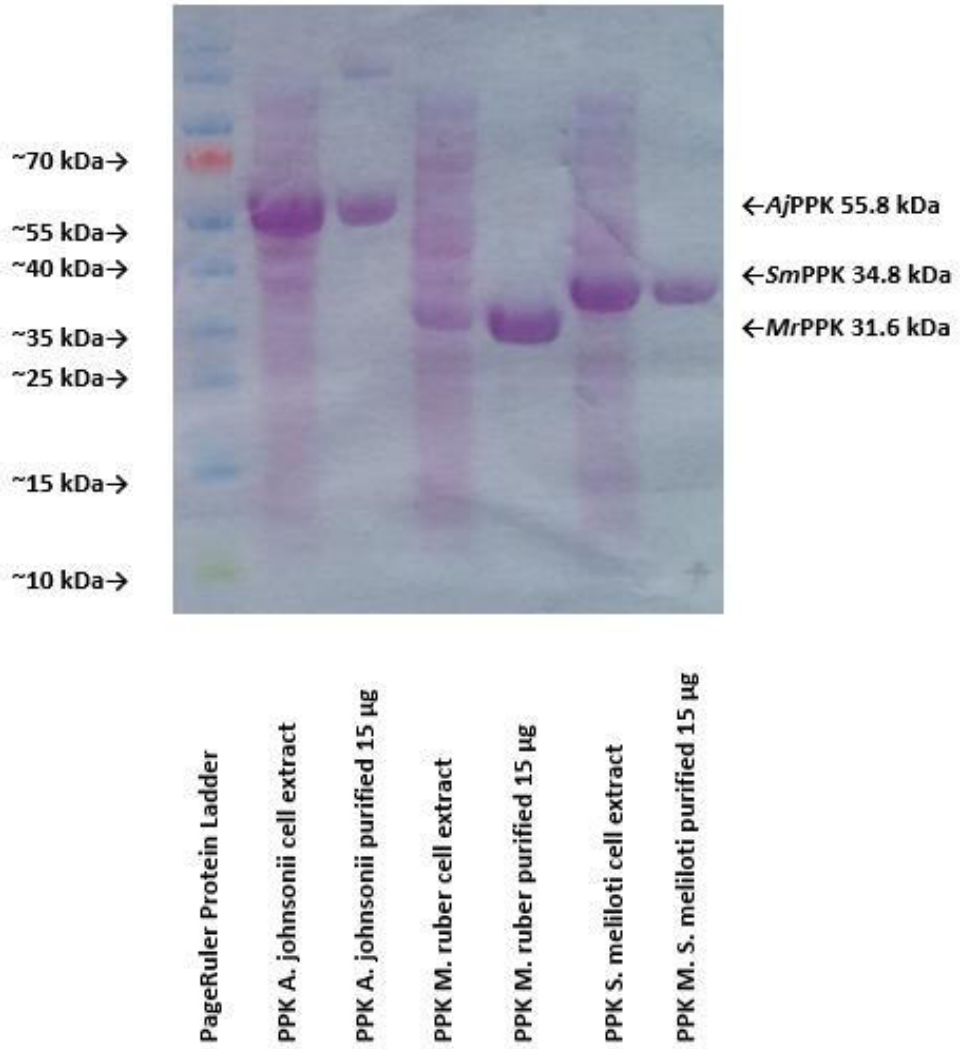


Figure 6: SDS-PAGE of *Aj*PPK, *Mr*PPK and *Sm*PPK purified by Ni^{2+} -affinity chromatography

3.4.4. Protein Yields

Protein concentrations were measured photometrically at 280 nm by a NanoDrop device (Thermo Scientific). The respective concentrations and culture volumes were used to calculate the protein yields.

Table 11: Calculated protein yields of expressed enzymes

enzymes	***protein yield [mg L^{-1}]***
UMPK	5.1
AMPK	4.0
GMPK	39.1
CMPK	18.6
NDK	10.2
*Ec*PPK F1	3.2
*Ec*PPK F2	1.5
*Sm*PPK	10.5
*Aj*PPK	39.2
*Mr*PPK	7.4

3.5. HPLC methods

Nucleosides and their mono-, di and triphosphates, as well as NDP sugars, were analysed by C18 HPLC using a Kinetex™ C18 column in reversed phase ion-pairing mode and UV detection at 253 (guanine), 259 (adenine), 262 (uracil), 267 (thymine or 271 nm (cytosine). Two different methods were used for substrate and product quantifications, depending on initially applied substrates. Solutions containing only one nucleobase and polyP were separated as described by Bungaruang et al within 6.5 min long isocratic runs using 12.5% acetonitrile. Reaction solutions containing several nucleobases for NMPK and NDK tests were measured using 9% acetonitrile within 9 min long runs for better baseline separation.

3.6. General PPK conversions

3.6.1. Substrate specificity of all PPKs

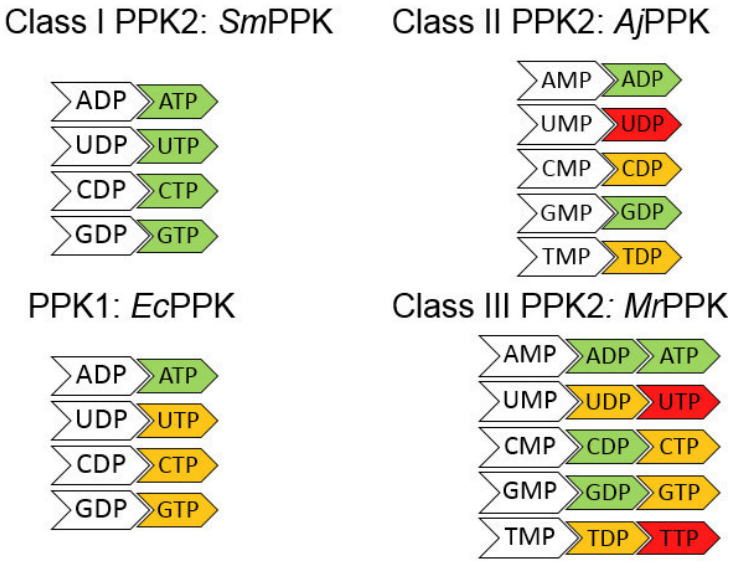
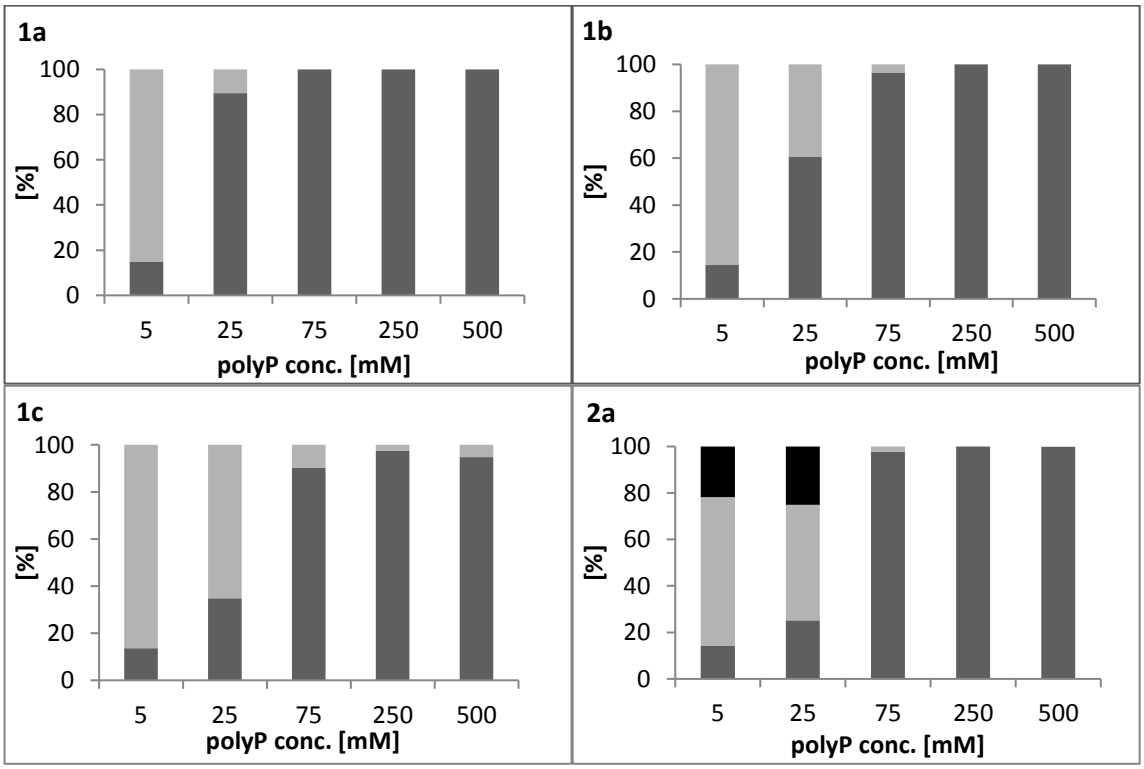


Figure 7: Substrate specificity test for all PPKs: 1 mM NXP, 5 mM polyP, 10 mM $MgCl_2$, 50 mM Tris, 50 µg mL^{-1} PPK, pH 7, 30°C; red: 0%, yellow: < 10%, green: > 10% product formation; final conversion after 24 h

3.6.2. Determination of the appropriate polyP concentration for all PPKs



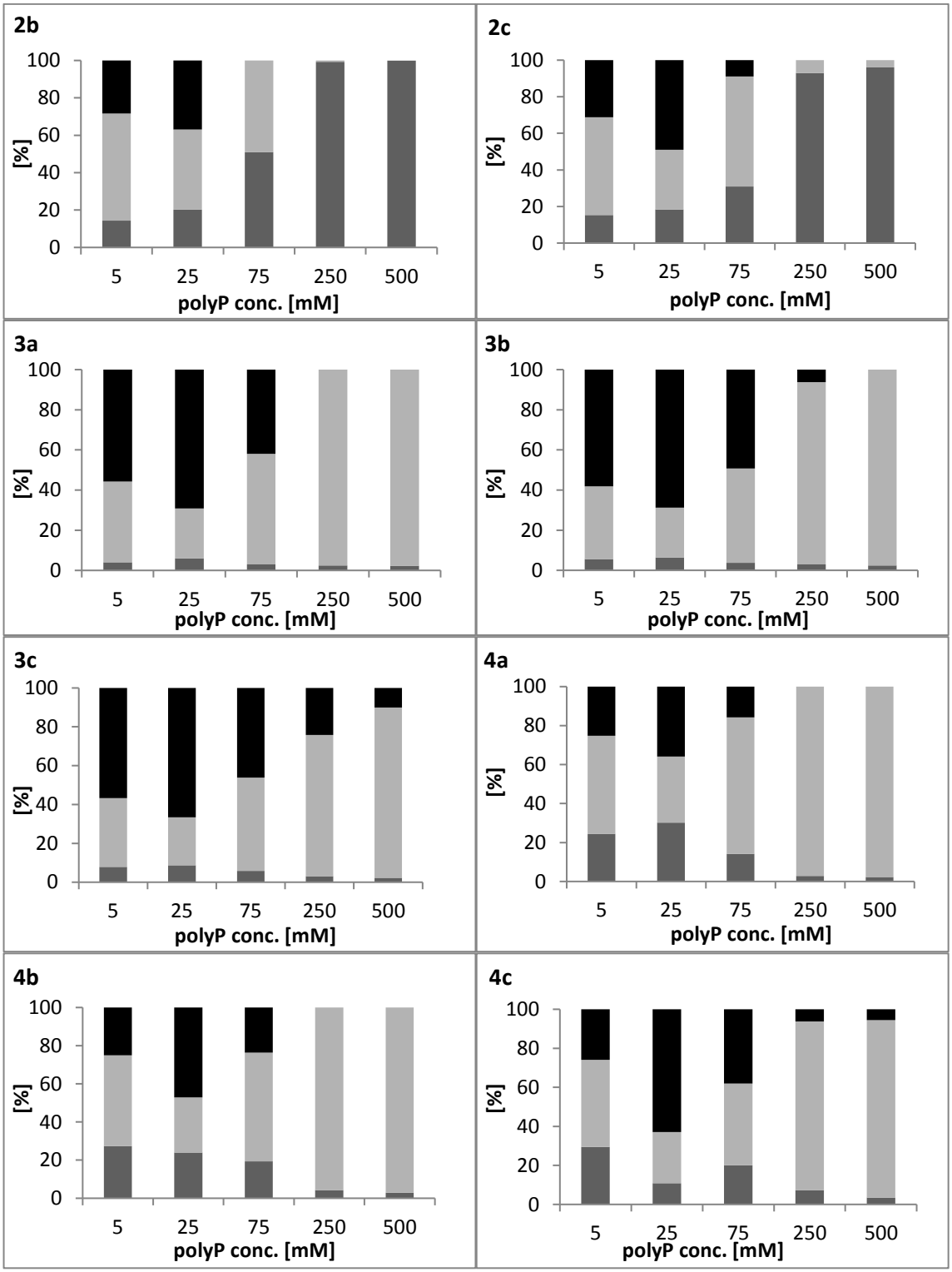


Figure 8: Determination of the appropriate polyP concentration for conversion with all PPKs: 1a-c: *Aj*PPK, 2a-c: *Mr*PPK, 3a-c: *Sm*PPK, 4a-c: *Ec*PPK; a: after 1 hour, b: after 6 hours, c: after 24 hours; 1-2: 1 mM AMP, 3-4: 1 mM ADP; grey: AMP, dark grey: ADP, black ATP; 10 mM $MgCl_2$, 50 mM Tris, 50 µg mL^{-1} PPK, pH 7, 30°C; varying polyP conc. (5, 25, 75, 250, 500 mM)

3.6.3. Alternative phosphate-donors: pyrophosphate and sodium metaphosphate

No reaction was observed with 10 mM AMP, 25 mM $MgCl_2$, 250 mM sucrose, 0.1 mg mL^{-1} BSA and 12.5 mM pyrophosphate (PPi) or 4 mM sodium metaphosphate (50 µg mL^{-1} *Ac*SuSy, 100 µg mL^{-1} *Mr*PPK, pH 5.5, 45°C).

3.6.4. Reverse reaction of *Mr*PPK and *Sm*PPK

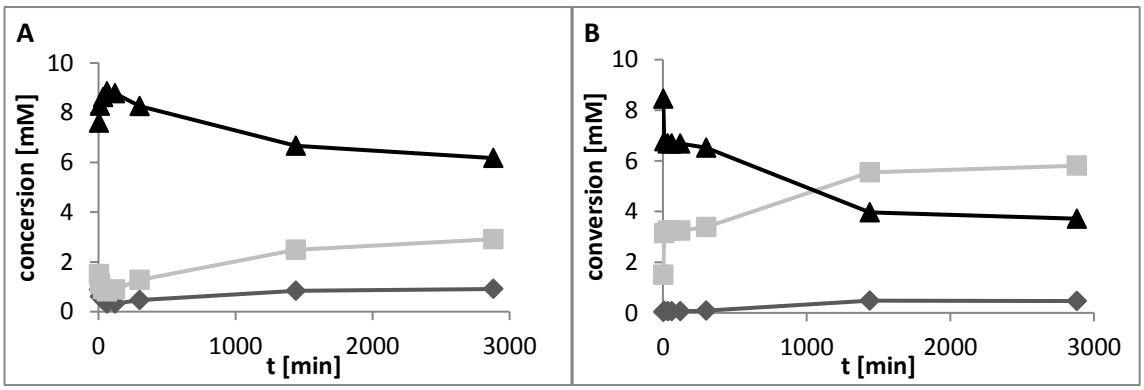


Figure 9: Reverse reaction of *Mr*PPK and *Sm*PPK: A: *Mr*PPK, B: *Sm*PPK; 10 mM ADP, 1 mM polyP, 25 mM $MgCl_2$, 50 mM MES, 0.1 mg mL^{-1} BSA, pH 5.5, 45°C, 100 µg mL^{-1} PPK; grey: AMP, dark grey: ADP, black: ATP

3.6.5. Coupling reactions of *Aj*PPK and *Sm*PPK

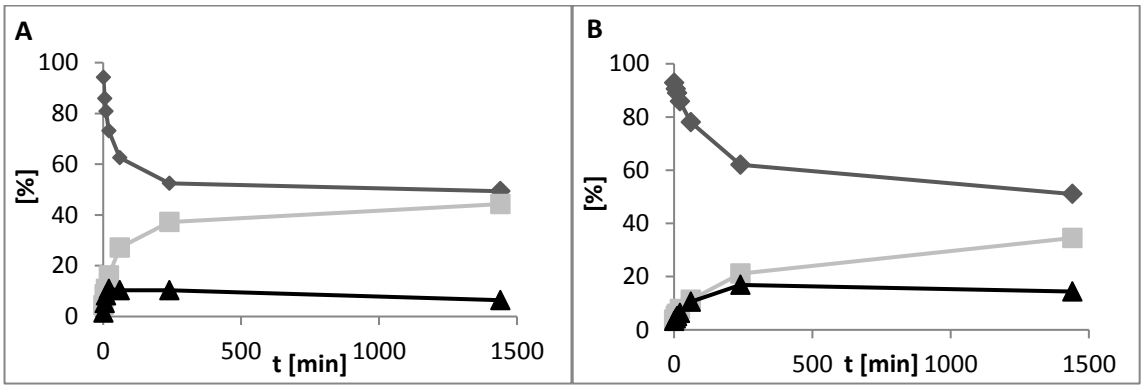


Figure 10: Coupling of *Aj*PPK and *Sm*PPK with two different $MgCl_2$ conc.: A: 100 mM $MgCl_2$, B: 10 mM $MgCl_2$;10 mM AMP, 25 mM polyP, 50 mM Tris, pH 8, 37°C, 200 µg mL^{-1} *Aj*PPK, 50 µg mL^{-1} *Sm*PPK; grey: AMP, dark grey: ADP, black ATP

Similar results were obtained with GMP and UMP as substrates. 100 mM $MgCl_2$ gave higher conversions in all reactions. GMP was converted worse than AMP at both $MgCl_2$ conc. UMP was just converted with 100 mM $MgCl_2$.

3.7. NMPK conversions

3.7.1. Pre-test with AMPK, CMPK, GMPK and UMPK

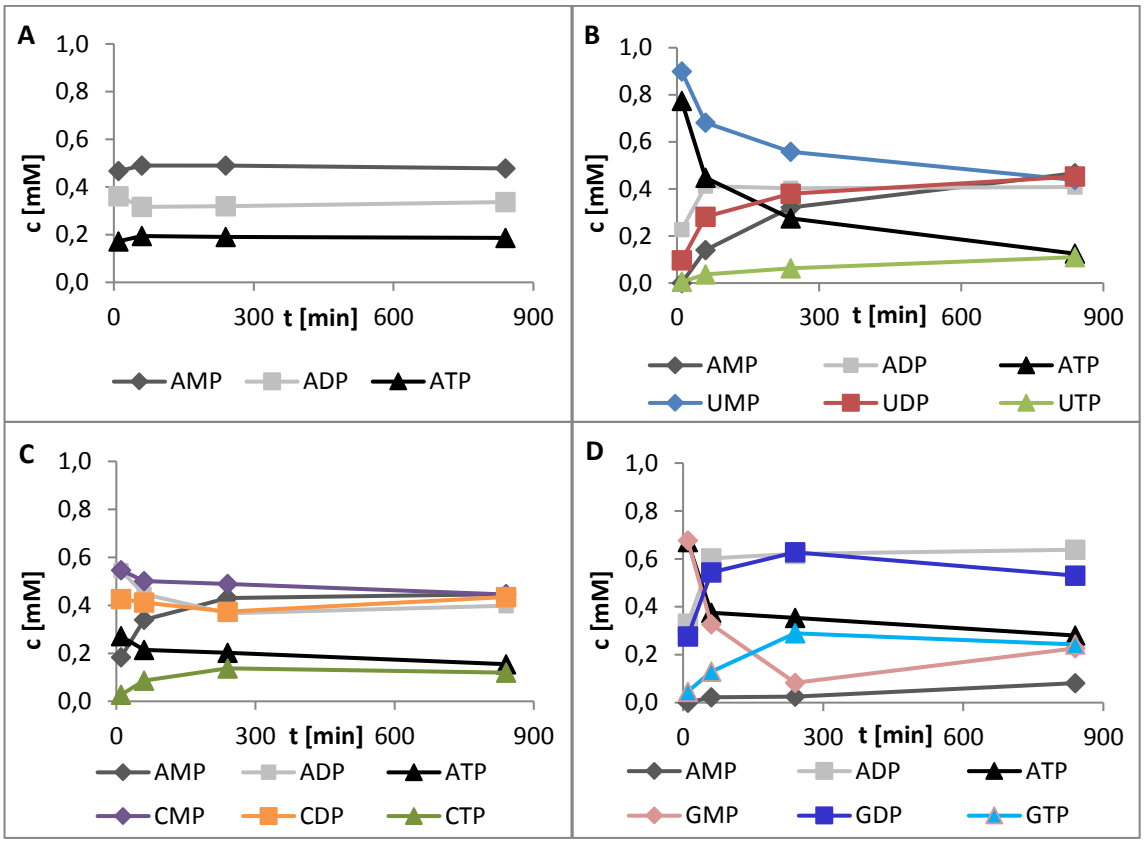


Figure 11: Pre-test with different NMP-Ks: A: AMPK, B: UMPK, C: CMPK, D: GMPK; 1mM NMP, 1 mM ATP, 10 mM $MgCl_2$, 0.1 mg mL-1 BSA, pH 7.6, 30°C, 100 µg mL^{-1} NMP-K; grey: AMP, dark grey: ADP, black ATP

3.7.2. UMPK conversion with UTP as alternative phosphate donor

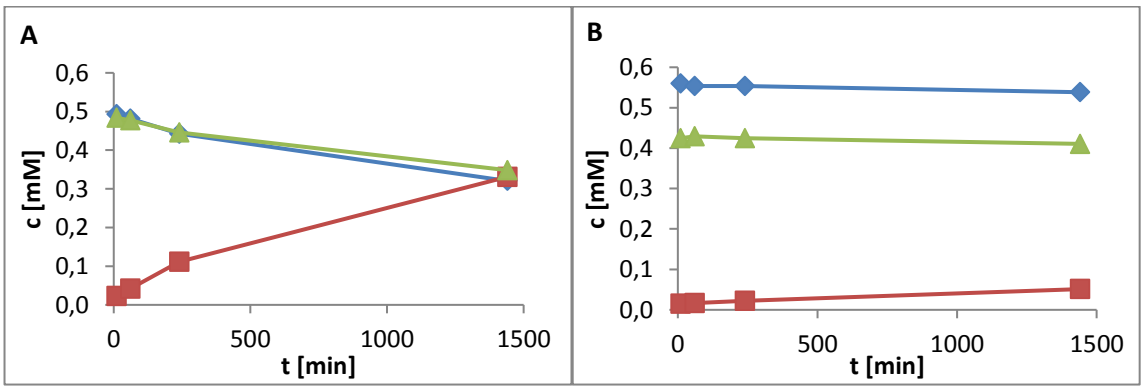


Figure 12: Conversion of UMP with UTP as alternative phosphate donor and two different enzyme concentrations: A: 500 µg mL^{-1} UMPK, B: 50 µg mL^{-1} UMPK, 1 mM UMP, 1 mM UTP, 10 mM MgCl2, 50 mM Tris, 0.1 mg mL^{-1}, pH 7.6, 30°C; 50 or 500 µg mL^{-1}; blue: UMP, red: UDP, green: UTP

Table 12: UMPK activities measured for UMP conversion

	activity [mU/mg]		
enzyme conc.	***UMP***	***UDP***	***UTP***
500 µg mL^{-1}	-0,0023	0,0083	-0,0018
50 µg mL^{-1}	-0,0027	0,0100	-0,0023

3.7.3. UMPK conversion with UTP as alternative phosphate donor and different enzyme concentrations

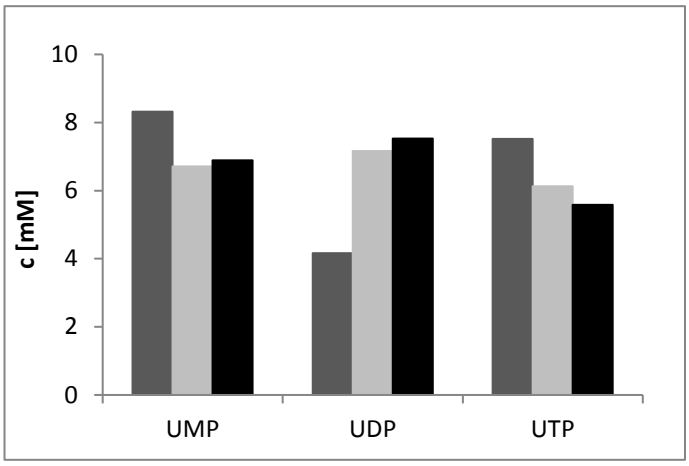


Figure 13: Conversion of UMP with different UMPK concentrations: 10 mM UMP, 10 mM UTP, 10 mM $MgCl_2$, 0.1 mg mL^{-1} BSA, 50 mM Tris, pH 7.6, 30°C; 100, 250 or 500 µg mL^{-1} UMPK; dark grey: 100 µg mL^{-1}, grey: 250 final µg mL^{-1}, black: 500 µg mL^{-1}; conversion after 48 h

3.7.4. UMPK conversion with diff. UTP concentrations

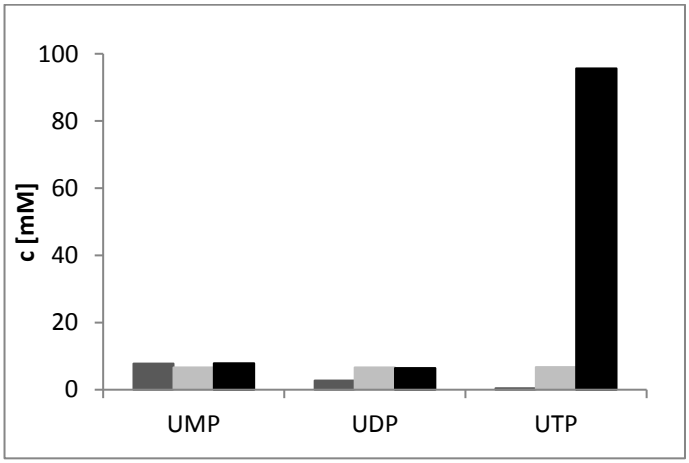


Figure 14: Conversion of UMP by UMPK and diff. UTP concentrations: 10 mM UMP, 10 mM $MgCl_2$, 50 mM Tris, 0.1 mg mL^{-1} BSA, pH 7.6, 30°C, 500 µg mL^{-1} UMPK; dark grey: 2 mM UTP, grey: 10 mM UTP, black: 100 mM UTP

3.7.5. Cascade reaction of UMPK and *Gm*SuSy at 3 diff. pH conditions

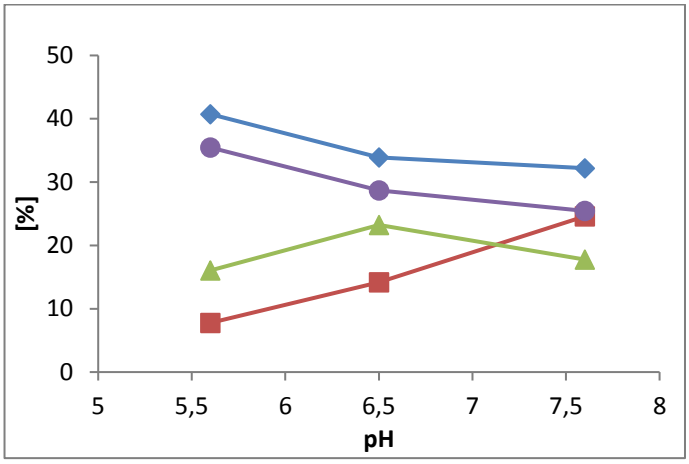


Figure 15: pH profiles of UMP conversion by UMPK and GmSuSy: 10 mM UMP, 10 mM UTP, 10 mM $MgCl_2$, 0.1 mg mL^{-1} BSA, 250 mM sucrose, pH 5.6, 6.5, 7.6; 250 µg mL^{-1} UMPK, 50 µg mL^{-1} *Gm*SuSy; blue: UMP, red: UDP, green: UTP, purple: UDP-glc; conversion after 24 h

3.8. ATP Regeneration for UDP-glc synthesis

3.8.1. Coupling of UMPK and *Gm*SuSy with *Mr*PPK or *Sm*PPK

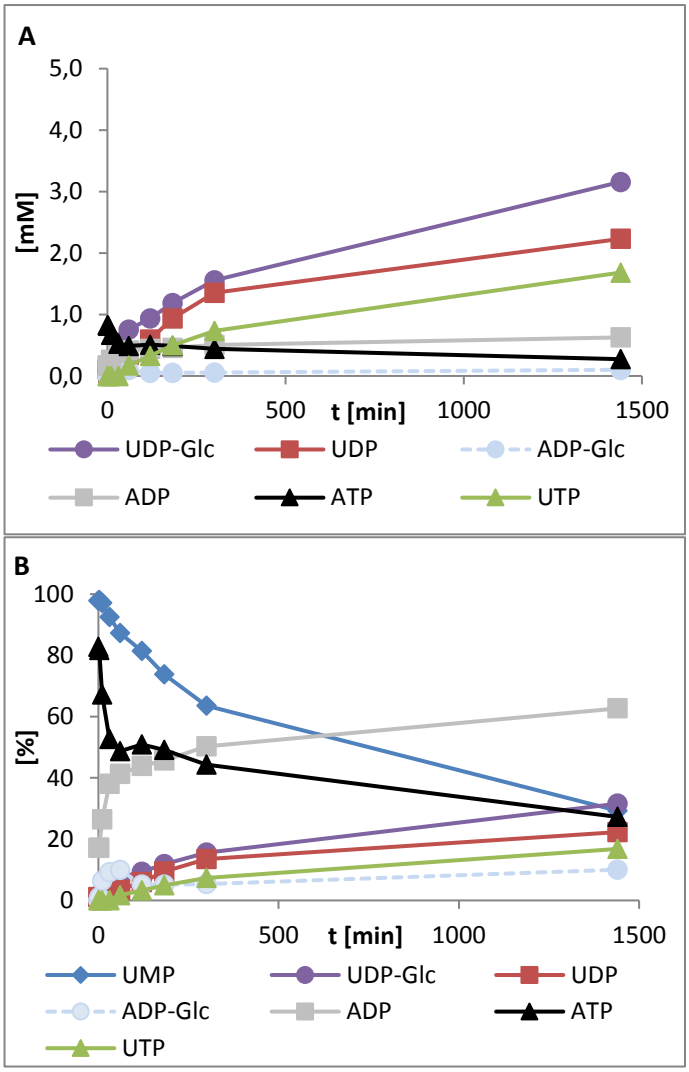


Figure 16: Conversion of UMP by *Sm*PPK, UMPK and *Gm*SuSy: 10 mM UMP, 1 mM ATP, 25 mM $MgCl_2$, 0.1 mg mL^{-1} BSA, 20 mM polyP, 250 mM sucrose, 50 mM MES, pH 6.0, 30°C; 50 µg mL^{-1} *Gm*SuSy, 100 µg mL^{-1} UMPK, 100 µg mL^{-1} *Sm*PPK; A: conversion in mM, B: conversion in %

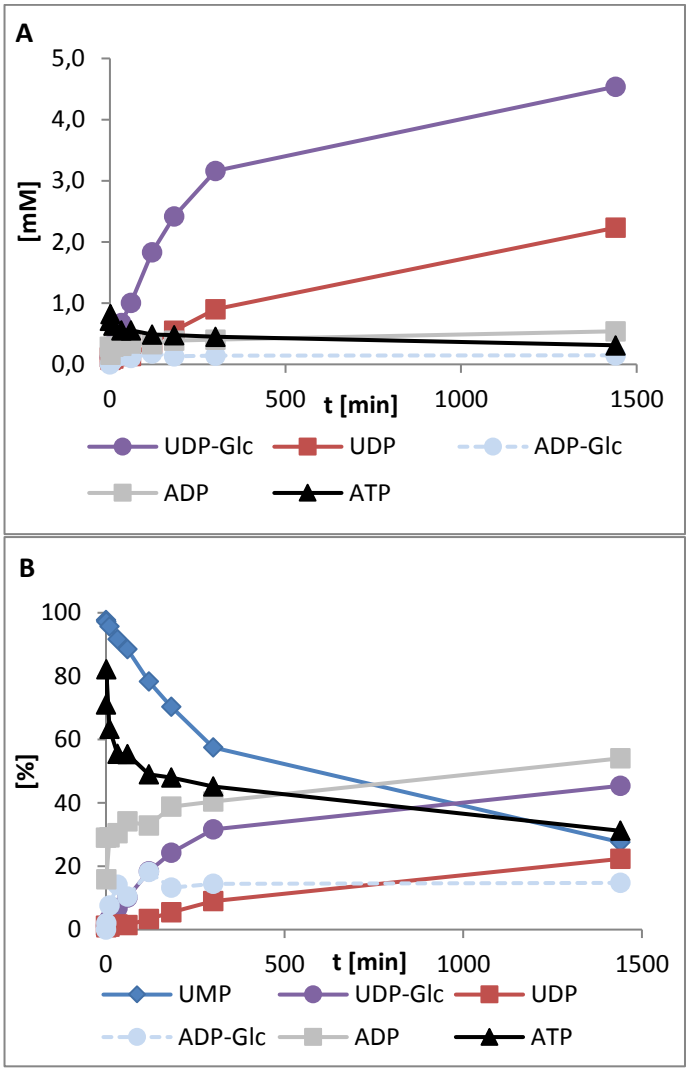


Figure 17: Conversion of UMP by *Mr*PPK, UMPK and *Gm*SuSy: 10 mM UMP, 1 mM ATP, 25 mM $MgCl_2$, 0.1 mg mL^{-1} BSA, 20 mM polyP, 250 mM sucrose, 50 mM MES, pH 6.0, 30°C; 50 µg mL^{-1} *Gm*SuSy, 100 µg mL^{-1} UMPK, 100 µg mL^{-1} *Mr*PPK, A: conversion in mM, B: conversion in %

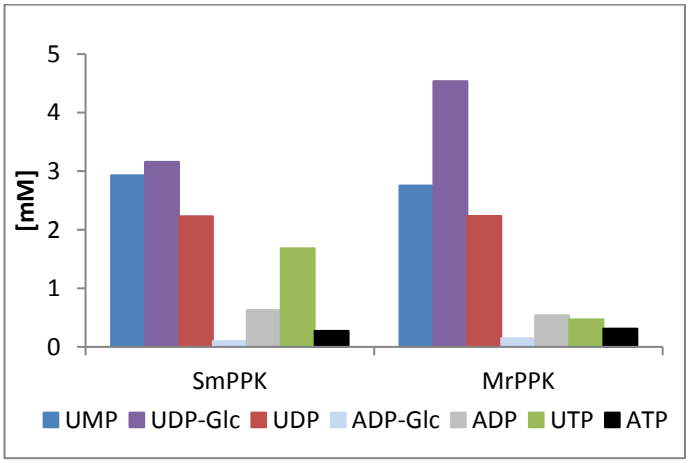


Figure 18: Conversion of UMP by *Mr*PPK or *Sm*PPK, respectively, UMPK and *Gm*SuSy: 10 mM UMP, 1 mM ATP, 25 mM $MgCl_2$, 0.1 mg mL^{-1} BSA, 20 mM polyP, 250 mM sucrose, 50 mM MES, pH 6.0, 30°C; 50 µg mL^{-1} *Gm*SuSy, 100 µg mL^{-1} UMPK, 100 µg mL^{-1} PPK

3.8.2. Investigation of GTP as effector for coupling of UMPK, *Gm*SuSy and *Mr*PPK

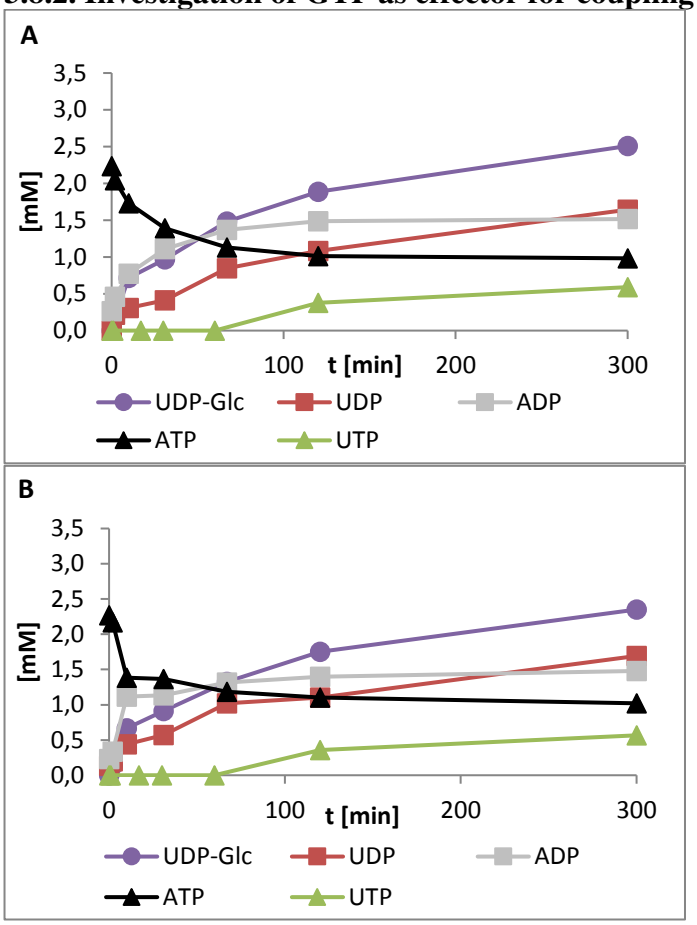


Figure 19: Conversion of UMP by UMPK, *Gm*SuSy and *Mr*PPK with and without GTP as effector: A: without GTP, B: with GTP; 10 mM UMP, 2.5 mM ATP, 25 mM $MgCl_2$, 0.1 mg mL^{-1} BSA, 250 mM sucrose, 50 mM MES, pH 6.5, 100 µg mL^{-1} *Gm*SuSy, 100 µg mL^{-1} *Mr*PPK, 100 µg mL^{-1} UMPK; 0 or 0.3 mM GTP

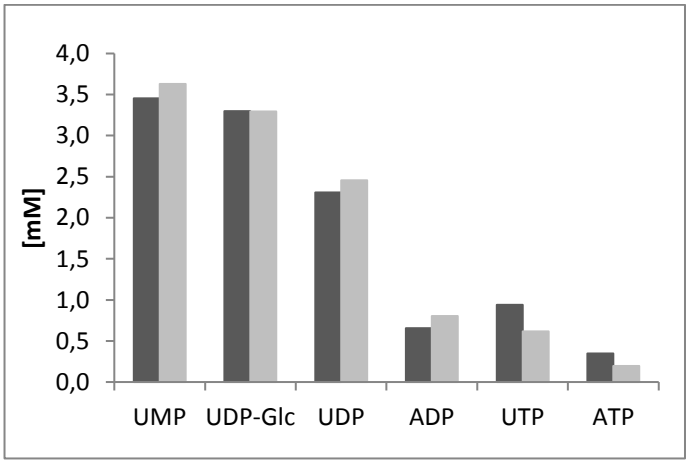


Figure 20: Conversion of UMP by UMPK, *Gm*SuSy and *Mr*PPK with 0.3 mM GTP and without GTP after 24 h; dark grey: 0 mM GTP, grey: 0.3 mM GTP

3.8.3. Increasing substrate concentrations for UDP-glc synthesis with UMPK, *Gm*SuSy and *Mr*PPK

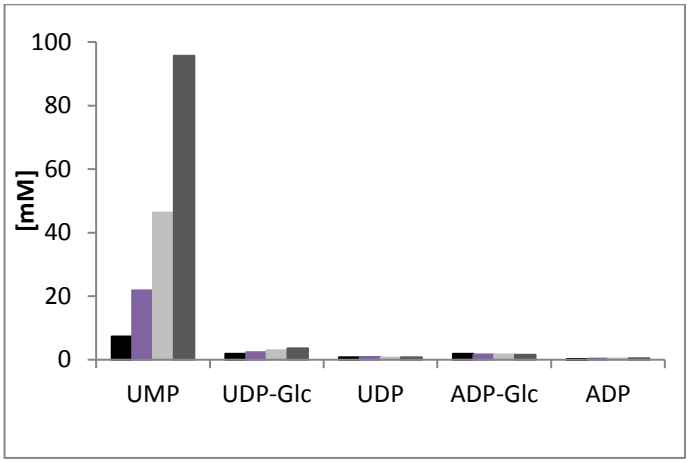


Figure 21: Conversion of UMP by UMPK, *Gm*SuSy and *Mr*PPK with varying substrate concentrations (10-100 mM UMP): 2 mM ATP, 25 mM $MgCl_2$, 0.1 mg mL^{-1} BSA, 200 mM polyP, 750 mM sucrose, 50 mM MES, 30°C, pH 6, 300 µg mL^{-1} *Gm*SuSy, 300 µg mL^{-1} UMPK, 300 µg mL^{-1} *Mr*PPK; 10, 25, 50 or 100 mM UMP; black: 10 mM UMP, purple: 25 mM UMP, grey: 50 mM UMP, dark grey: 100 mM UMP

Table 13: Conversion of diff. UMP concentrations by UMPK, *Gm*SuSy and *Mr*PPK after 24 h

	conversion [mM] after 24 h				
substrate conc.	***UMP***	***UDP-glc***	***UDP***	***ADP-glc***	***ADP***
10 mM UMP	7,31	1,90	0,79	1,89	0,11
25 mM UMP	21,78	2,40	0,82	1,64	0,36
50 mM UMP	46,29	3,01	0,70	1,63	0,37
100 mM UMP	95,63	3,61	0,77	1,52	0,48

3.9. NDK conversions

3.9.1. NDK conversions with UDP, CDP, GDP and dTDP as substrates

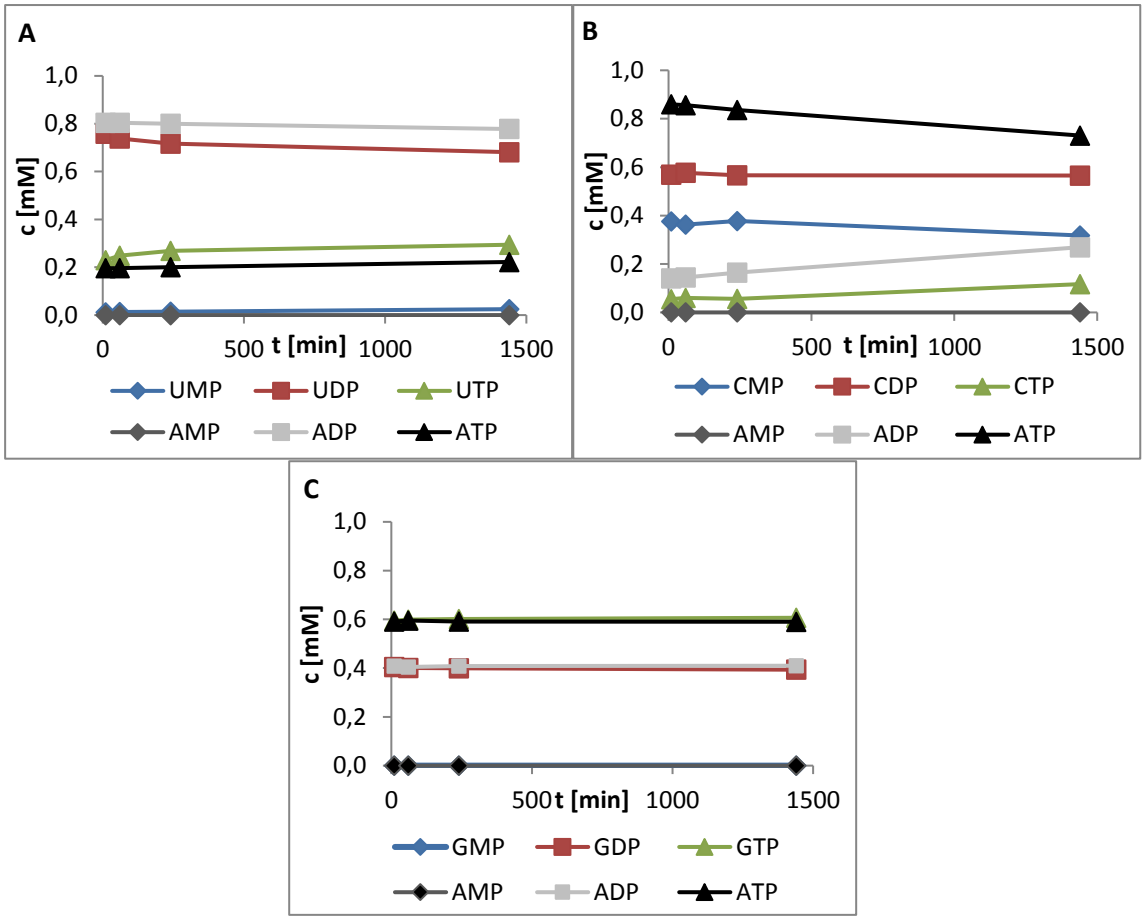


Figure 22: Conversions of UDP, CDP, and GDP by NDK; A: UDP, B: CDP, C: GDP; 1 mM NDP, 1 mM ATP, 10 mM $MgCl_2$, 50 mM Tris, 0.1 mg mL^{-1} BSA, pH 7.6, 30°C 100 µg mL^{-1} NDK

Separation of AMP and GMP was not possible with the applied HPLC method. Also dTXP and AXP could not be detected separately.

3.10. Gene sequences

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identical to Sequence from Escherichia coli strain BL21 (TaKaRa): GenBank AIA55343.1.
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CTGCTGAAAGCGCAGATCGCTCACTTCTTCGAGCACTACAAAGACCTCGAAAAAG
GCAAGTGGGTGAAAGTTGAAGGTTGGGAAAACGCAGAAGCCGCTAAAGCTGAAA
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AMPK:
identical to Sequence from Escherichia coli strain BL21 (TaKaRa): GenBank AAB40228.1
Protein ID: AJH09378.1

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GCAAACTGGTCACCGACGAACTGGTGATCGCGCTGGTTAAAGAGCGCATTGCTCA
GGAAGACTGCCGTAATGGTTTCCTGTTGGACGGCTTCCCGCGTACCATTCCGCAGG
CAGACGCGATGAAAGAAGCGGGCATCAATGTTGATTACGTTCTGGAATTCGACGT
ACCGGACGAACTGATTGTTGATCGTATCGTAGGCCGCCGCGTTCATGCGCCGTCTG
GTCGTGTTTATCACGTTAAATTCAATCCGCCGAAAGTAGAAGGCAAAGACGACGT
TACCGGTGAAGAACTGACTACCCGTAAAGACGATCAGGAAGAAACCGTGCGTAA
ACGTCTGGTTGAATACCATCAGATGACTGCACCGCTGATCGGCTACTACTCCAAA
GAAGCGGAAGCGGGTAACACCAAATACGCGAAAGTTGACGGCACCAAGCCGGTT
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UMPK:
identical to Sequence from Escherichia coli strain BL21 (TaKaRa): GenBank EMV48922.1
Protein ID: AJH09128.1

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TGATGCACTGCACCGCGCCTATGTGAACGCTCGTCTGATGTCCGCTATTCCATTGA
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CAGCTTGCCTGCGTGGTATCGAAATTGAAGCCGATGTGGTGCTGAAAGCAACCAA
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GAGCAACTGACTTACAGCGAAGTGCTGGAAAAAGAGCTGAAAGTCATGGACCTG
GCGGCCTTCACGCTGGCTCGTGACCATAAATTACCGATTCGTGTTTTCAATATGAA

CAAACCGGGTGCGCTGCGCCGTGTGGTAATGGGTGAAAAAGAAGGGACTTTAATC
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CMPK:
identical to Sequence from Escherichia coli strain BL21 (TaKaRa): GenBank KFH76471.1
Protein ID: AJH09806.1

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CAGAAATTGGCTCTCGCATAA

***Aj*PPK**
identical to Sequence from Escherichia coli strain BL21 (TaKaRa): GenBank
Protein ID: BAC76403.1

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***Sm*PPK**
identical to Sequence from Escherichia coli strain BL21 (TaKaRa): HG799420.1
Protein ID: DL73027.1

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***Mr*PPK**

identical to Sequence from Escherichia coli strain BL21 (TaKaRa): GenBank AGK05310.1
Protein ID: n.a.

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GAGTAA

3.11. pET-28a vector

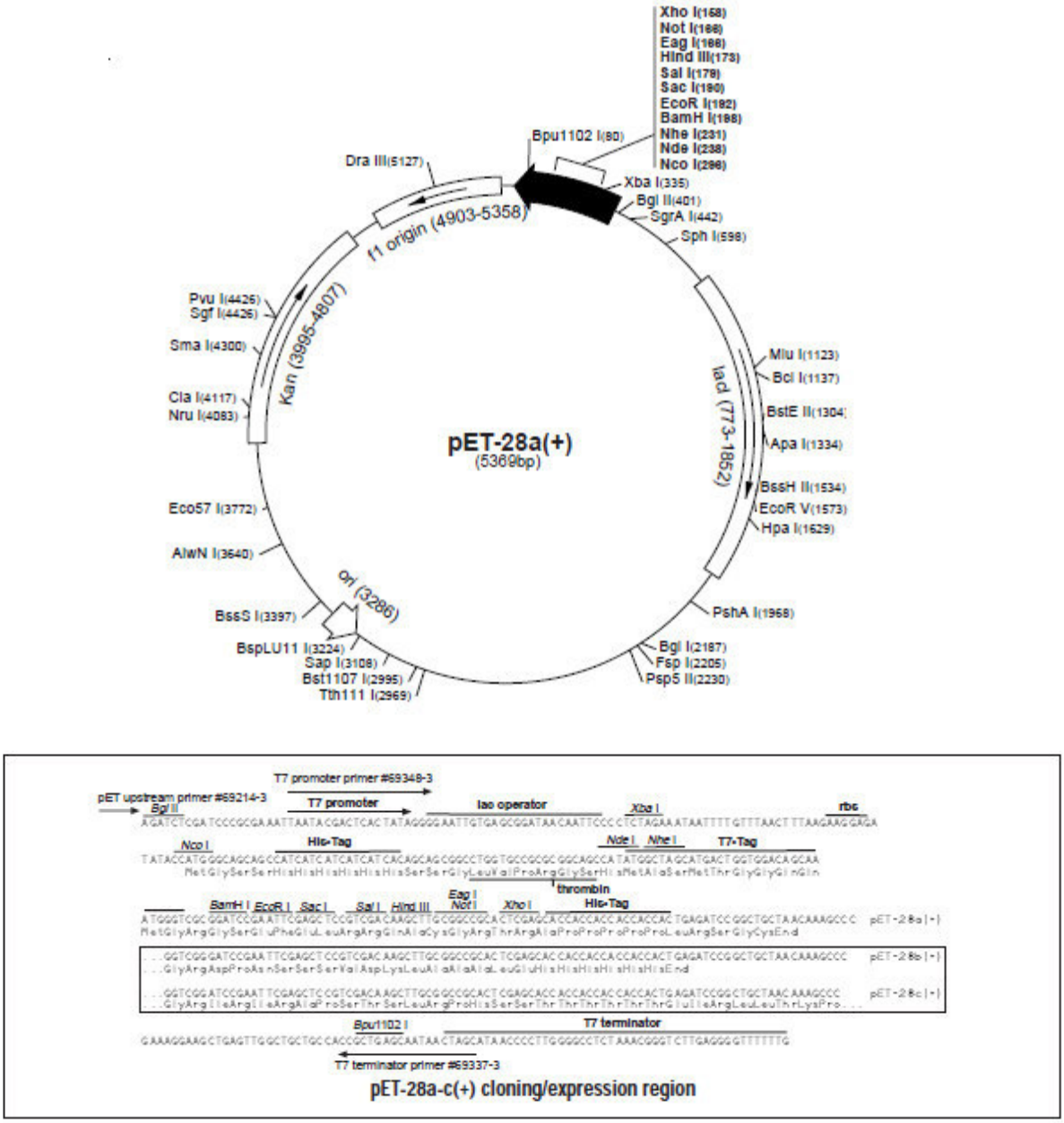

Figure 23: pET-28a vector used for cloning work

3.12. List of tables

3.13. List of figures

3.14. References

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[2] J. Quan und J. Tian, „Circular Polymerase Extension Cloning of Complex Gene Libraries and Pathways," *PLoS ONE* **2009**, *7*, 1-6.