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Improving the Phosphatase-Catalyzed Synthesis of 5′-Nucleotides: A Reaction Engineering Approach

Marina S. Robescu [1], Teodora Bavaro [1], Fabrizio Medici [2], Giovanna Speranza [2,*], Daniela Ubiali [1] and Marco Rabuffetti [2,*]

[1] Department of Drug Sciences, University of Pavia, Viale Taramelli 12, 27100 Pavia, Italy; marinasimona.robescu@unipv.it (M.S.R.); teodora.bavaro@unipv.it (T.B.); daniela.ubiali@unipv.it (D.U.)
[2] Department of Chemistry, University of Milan, Via Golgi 19, 20133 Milan, Italy; fabrizio.medici@unimi.it
* Correspondence: giovanna.speranza@unimi.it (G.S.); marco.rabuffetti1@unimi.it (M.R.); Tel.: +39-02-503-14097 (G.S.); +39-02-503-14100 (M.R.)

Abstract: 5′-Phosphorylation of nucleosides is a reaction as important in nature and in industry as it is cumbersome to be performed. Whilst chemical phosphorylation relies on the use of harsh reagents, solvents, and conditions, as well as on the need for protection–deprotection steps, biocatalysis can be a tool to achieve one-step phosphorylation reactions, which are selective, protecting group-free, and occurring under mild and sustainable conditions. In this work, the wild-type non-specific acid phosphatase from *Morganella morganii* (PhoC-*Mm*) was expressed, purified, and used for the synthesis of inosine 5′-monophosphate (IMP), an important food additive, by using pyrophosphate (PPi) as an inexpensive phosphate donor in a fully aqueous medium at 30 °C. Via the fine-tuning of the reaction set-up taking into account the type of buffer, amount of PPi, mode/time of PPi addition, and enzyme and substrate concentration, PhoC-*Mm* could be used for catalyzing the phosphorylation of inosine (I) to IMP in a good yield and high purity (62% yield). The catalysis of the hydrolytic reaction direction, which is the primary function of phosphatases in nature, was here reversed to a certain extent by a reaction engineering approach, without the need for protein engineering strategies.

Keywords: non-specific acid phosphatase (NSAP) from *Morganella morganii*; wild-type enzyme; inosine 5′-monophosphate (IMP); enzymatic phosphorylation; transphosphorylation; fed-batch; disodium pyrophosphate dibasic; sustainable synthesis; regioselectivity; reaction engineering

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1. Introduction

Phosphate monoester motifs are widespread in nature and are present in molecules involved in many biological pathways [1]. Among them, nucleotides represent a preeminent class due to the central role they play in almost all cellular processes [2,3]. In addition, their synthetic analogs (nucleotide analogs) are the fundamental agents of several anti-cancer and anti-viral drugs as well as the water-soluble prodrugs of pharmacologically active nucleosides [4,5]. Finally, some natural nucleotides such as inosine 5′-monophosphate (IMP) and guanosine 5′-monophosphate (GMP) are used as flavor enhancers in savory food manufacturing due to their ability to synergistically elicit the perception of umami taste in combination with monosodium glutamate (MSG) [6,7]. For these reasons, considerable efforts have been directed toward the selective synthesis of naturally occurring nucleotides and their analogs [5,8].

The most straightforward way to prepare nucleoside 5′-monophosphates consists of the introduction of a phosphate group at the 5′-hydroxyl position of their parent nucleosides mainly by reaction with pentavalent phosphoryl donors such as phosphoryl chloride ($POCl_3$) in the presence of trialkyl phosphates as solvents. Such a process is quite laborious due to the toxicity and moisture sensitivity of the reagents as well as the extensive formation of by-products. In addition, the harsh reaction conditions generally fail to preserve most acid-labile

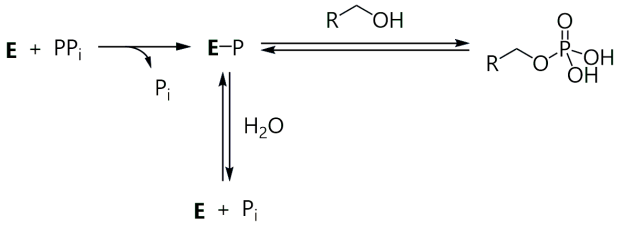
moieties [9,10]. Owing to these disadvantages, different phosphate donors or alternative strategies, i.e., formation of trivalent phosphite followed by oxidation [11–13], have been developed to allow milder and more selective syntheses. These methods, however, are not able to avoid drawbacks such as the need for multi-step sequences required by protecting group chemistry together with poor selectivity and moderate/low yields. Thus, more ecofriendly and sustainable routes to 5′-monophosphate nucleosides are highly desirable.

Biocatalytic procedures have emerged in the last few decades as viable alternatives to conventional chemical routes, matching high selectivity and mild conditions as well as generating less waste in agreement with "green chemistry" principles [14,15]. Among enzymes from the nucleotide metabolism, deoxyribonucleoside kinases (dNKs, E.C. 2.7.1) catalyze the selective and irreversible phosphorylation of nucleoside 5′-position using ATP as a high-energy phosphate donor [16,17]. However, examples of dNK-based phosphorylation are limited to date due to the relatively low stability and high cost of ATP, as well as the inhibitory effect of the accumulating ADP [18,19]. In order to allow the use of ATP in catalytic amounts, several recycling systems have been developed to turn ADP into ATP, thus making the process less expensive [20,21].

Alternatively, the formation of a new P-O bond can be catalyzed by phosphatases, a class of enzymes that dephosphorylate in vivo a large set of phosphate esters, but that are also able to perform the reverse reaction (transphosphorylation reaction) [16,22]. In particular, non-specific acid phosphatases (NSAPs), widely distributed among enteric bacteria, exhibit optimal catalytic activity at acidic to neutral pH values and are characterized by a very broad substrate specificity being active toward different and structurally unrelated phosphoesters such as nucleotides and sugar phosphates [17,22,23].

In the late 1990s, an NSAP from *Morganella morganii* NCIMB10466 strain (PhoC-*Mm*) possessing a regioselective phosphotransferase activity specific for the 5′-position of nucleosides was described by Asano et al. who exploited it for the selective phosphorylation of C-5′ of inosine (I) to produce IMP, using the food additive pyrophosphate (PPi, E450 as sodium salt) as the phosphate donor [24,25].

Kinetic data indicate that the key step in the NSAP-catalyzed reaction is the formation of a phosphate–enzyme intermediate (**E**-P), which undergoes either nucleophilic attack by a substrate hydroxyl group to form the phosphorylated product (transphosphorylation) or hydrolysis, releasing inorganic phosphate (Pi) in the reaction medium. In addition, due to the reversibility of the phosphate transfer, a progressive dephosphorylation of the formed phosphate ester may occur (Scheme 1) [17,26].



Scheme 1. Mechanism of NSAP-catalyzed transphosphorylations and hydrolysis.

Thus, in the case of the PhoC-*Mm*-mediated phosphorylation of inosine (I) to inosine 5′-monophosphate (IMP) in the presence of PPi, a maximum 41% conversion was observed due to the high hydrolytic activity of the wild-type catalyst toward the synthesized IMP [25]. In order to reduce the dephosphorylating activity and to increase the efficiency of the transphosphorylation reaction, a mutated acid phosphatase was identified (I171T-G92D PhoC-*Mm*) with more advantageous kinetic parameters responsible for the improved productivity ($K_{m\ inosine}$ = 117 mM vs. 42.6 mM for wild-type and mutant enzyme, respectively) [27].

Later, a number of acid phosphatases from enteric bacteria with regioselective PPi-nucleoside phosphotransferase activity were isolated, cloned, and used for the synthesis of

IMP [28,29]. For all the investigated bacteria, the IMP productivities were inferior to those of the enzyme from *M. morganii* (PhoC-*Mm*) even in the case of mutant acid phosphatases [30].

However, some reaction details for this industrially relevant enzymatic phosphorylation are still lacking and the influence of the reaction parameters upon nucleotide production has never been deeply investigated. It is worth noting that, not unlike conventional chemical routes, enzymatic strategies are hampered by many potential bottlenecks and need fine-tuning as well. Therefore, applying the approach of reaction engineering, e.g., investigating multiple reactions at varied parameters, can help overcome these difficulties and additional benefits such as the shift of unfavorable equilibria can be obtained. Indeed, reaction engineering has long provided an effective tool for bioprocess development, resulting in cost-effective and scalable methodologies [31].

In this study, the fed-batch phosphorylation of inosine to IMP using the wild-type Phoc-*Mm* as the biocatalyst and disodium pyrophosphate (PPi) as the phosphate source was investigated. The reaction conditions were optimized with respect to the type of buffer, amount of phosphate donor and mode/time of its addition, substrate concentration, and amount of added biocatalyst. Up to a 66% conversion and 62% of isolated yield of IMP was obtained. To the best of our knowledge, this represents the best result so far observed with a wild-type non-specific acid phosphatase.

2. Materials and Methods

2.1. Reagents and Equipment

All solvents and reagents were purchased from Merck (Milano, Italy) and were used without further purification.

Freeze-drying was performed using a Telstar LyoQuest (Azbil Telstar, Barcelona, Spain) freeze-dryer.

The expression vector pET28a(+) containing the PhoC-*Mm* gene was kindly provided by Prof. Kurt Faber (University of Graz, Graz, Austria). The protein was purified by fast protein liquid chromatography (FPLC) using a GE ÄKTA Purifier (Cytiva, Global Life Sciences Solutions, Marlborough, MA, USA) 100 FPLC System w/UPC-900 (HisTrap™ High Performance 1 mL pre-packed columns (Cytiva, Global Life Sciences Solutions, Marlborough, MA, USA)).

Analytical HPLC was performed using a JASCO LC-4000 chromatographer equipped with a UV-4070 UV–Vis detector (JASCO Applied Sciences, Halifax, NS, Canada). Experimental conditions were as follows: column, Jupiter 4 μM Proteo 90 Å (25 mm × 4.6 mm) (Danaher Corporation, Washington, DC, USA); wavelength, λ 254 nm; injection volume, 20 μL; flow rate, 1.00 mL/min; solvents, A = 0.1% TFA in water and B = ACN/0.1% TFA in water 80:20; gradient, 0–5.00 min: 95% A, 5.01–15.00: from 95% to 60% A, 15.01–15.05: from 60% to 95% A, 15.06–20.00: from 95% A.

Semi-preparative HPLC was performed using a Dionex Ultimate 3000 chromatographer equipped with a Dionex RS Variable Wavelenght detector (Thermo Fisher Scientific Inc., Waltham, MA, USA). Experimental conditions were as follows: column, Atlantis Prep T3 OBDTM 5 μm (19 mm × 100 mm) (Waters Corporation, Milford, MA, USA); wavelength, λ 254 nm; injection volume, 1 mL; flow rate, 10 mL/min; solvents, A = 0.1% TFA in water and B = ACN/0.1% TFA in water 80:20; gradient, 0–5.00 min: 100% A, 5.01–12.50: from 100% to 85% A, 12.01–15.00: from 85% to 0% A, 15.01–15.05: from 0% to 100% A.

^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded at 400, 100, and 161 MHz, respectively, on a Bruker AVANCE 400 spectrometer equipped with a TOPSPIN 3.7 software package (Bruker, Karlsruhe, Germany) at 300 K. D_2O (Merck) was used as solvent. ^{1}H and ^{13}C chemical shifts (δ) are given in parts per million (ppm) and referenced to the solvent signals (δ_H 4.79 ppm from tetramethylsilane (TMS) for D_2O); ^{31}P chemical shifts (δ) are referred to 85% H_3PO_4 as external standard (δ_P 0.00 ppm). Coupling constants (J) are reported in Hertz (Hz).

2.2. Expression and Purification of Recombinant PhoC-Mm

PhoC-*Mm* was expressed and purified following a slightly modified protocol [26].

Firstly, an overnight pre-culture was carried out in lysogeny broth medium (LB) (20 mL) containing kanamycin (50 µg/mL) at 37 °C and 180 rpm. Subsequently, a preparative culture was carried out in LB medium (1 L), and the cells were grown in a shaking incubator (180 rpm) at 37 °C to an optical density at 600 nm (OD_{600}) of 0.4–0.6. When the culture reached the desired OD, the flasks were transferred to 16 °C for 20 min, then the expression was induced by 0.2 mM IPTG (isopropyl β-D-1-thiogalactopyranoside), followed by shaking at 16 °C and 180 rpm overnight. The cells were harvested by centrifugation at 5000 rpm and 4 °C for 20 min. The wet cells (12 g) were resuspended in His-tag lysis buffer (50 mM Na-Pi pH 7.5 containing 300 mM NaCl and 10 mM imidazole; 24 mL) and were sonicated for 10 min. The disrupted cells were pelleted at 70,000 rpm and 4 °C for 45 min. The supernatant was purified using a Ni-NTA column at 4 °C by following the protocol of the manufacturer. The fractions containing the desired enzyme were identified by SDS-PAGE analysis, then combined, dialyzed against 20 mM NaOAc buffer pH 5.0, and finally concentrated. The concentration of the purified enzyme was measured by using the Bradford assay [32] and its activity was tested according to the standard spectrophotometric *p*-NPP assay [26].

2.3. PhoC-Mm Activity Assay

The standard activity assay (0.5 mL) was performed in 100 mM maleate buffer (pH 6.5) containing *p*-NPP (10 mM final concentration) and an appropriate amount of enzyme. The reaction was incubated at 34 °C and 70 rpm for 1 min. Afterwards, the reaction was quenched by the addition of 1 M NaOH (0.5 mL) and the absorbance of 4-nitrophenol (*p*-NP) was recorded at 405 nm (ε = 18,500 $M^{-1}cm^{-1}$). One unit of phosphatase activity (U) corresponds to the amount of *p*-NP (micromoles) released per minute under assay conditions.

2.4. Phosphorylation of Inosine Catalyzed by Phoc-Mm (Analytical Scale)

PhoC-*Mm* was added to a mixture of inosine (I) and PPi in 100 mM NaOAc buffer (pH 5.0, 500 µL). The resulting reaction was stirred at 30 °C. Concentrations and additions were set according to Tables 1–4 (see Figure 1 for the time course referred to Table 4). In the case of entries 4–7 (Table 2), the initial reaction was split into different portions after a proper amount of time for parallel screening. Aliquots (20 µL) were withdrawn at defined endpoints, diluted with 100 mM NaOAc buffer (pH 5.0, 180 µL), quenched with 1 M NaOH (20 µL), and analyzed by HPLC. Residual I and formed IMP percentages were calculated as follows: I (%) = ([I]/[I + IMP]) × 100 and IMP (%) = ([I]/[I + IMP]) × 100.

Table 1. Optimization of reaction parameters for the phosphorylation of I to IMP with 10 µg/mL PhoC-*Mm*: effect of the buffer type.

Entry [1]	Assessed Variable	I (mM)	PPi (mM)	Buffer	Time (h) [2]	IMP (%) [3]
1	reference reaction	40	250	NaOAc 100 mM pH 5.0	6	31
2	buffer	40	250	MES 100 mM pH 5.5	24	29
3	buffer	40	250	Sodium citrate 100 mM pH 5.0	6	14

[1] See Supplementary Materials (Paragraph S3.1) for graphs; [2] time was selected according to the highest conversion value; [3] the amount of inosine 5′-monophosphate (IMP), evaluated by HPLC, was calculated according to the following equation: IMP (%) = ([IMP]/[I + IMP]) × 100.

Table 2. Optimization of reaction parameters for the phosphorylation of I to IMP with 10 μg/mL PhoC-*Mm*: effect of the PPi concentration and mode of addition.

Entry [1]	Assessed Variable	I (mM)	PPi (mM)	Buffer	Time (h) [2]	IMP (%) [3]
1	reference reaction	40	250	NaOAc 100 mM pH 5.0	6	31
4	[PPi]	40	200	NaOAc 100 mM pH 5.0	6	21
5	[PPi]	40	400	NaOAc 100 mM pH 5.0	6	19
6	fed-batch PPi addition [4]	40	250	NaOAc 100 mM pH 5.0	6	9
7	fed-batch PPi addition [5]	40	500	NaOAc 100 mM pH 5.0	8	34
8	fed-batch PPi addition [6]	80	750	NaOAc 100 mM pH 5.0	9	44

[1] See Supplementary Materials (Paragraph S3.1) for graphs; [2] time was selected according to the highest conversion value; [3] the amount of inosine 5′-monophosphate (IMP), evaluated by HPLC, was calculated according to the following equation: IMP (%) = ([IMP]/[I + IMP]) × 100; [4] PPi was added progressively (41.7 mM/h for a total of 6 additions); [5] 250 mM PPi was added at t_0 and 6 h; [6] the following additions of PPi were made: (i) 250 mM at t_0; (ii) 125 mM at 3, 6, 24, and 27 h.

Table 3. Optimization of reaction parameters for the phosphorylation of I to IMP with 10 μg/mL PhoC-*Mm*: effect of inosine concentration.

Entry [1]	Assessed Variable	I (mM)	PPi (mM)	Buffer	Time (h) [2]	IMP (%) [3]
1	reference reaction	40	250	NaOAc 100 mM pH 5.0	6	31
9	[I] [4]	100	250	NaOAc 100 mM pH 5.0	6	22
10	[I] [5]	200	250	NaOAc 100 mM pH 5.0	6	18
11	[I] [6]	100	250	NaOAc 100 mM pH 5.0	6	29
12	[I]	80	250	NaOAc 100 mM pH 5.0	6	26

[1] See Supplementary Materials (Paragraph S3.1) for graphs; [2] time was selected according to the highest conversion value; [3] the amount of inosine 5′-monophosphate (IMP), evaluated by HPLC, was calculated according to the following equation: IMP (%) = ([IMP]/[I + IMP]) × 100; [4] I was completely solubilized after 1 h; [5] the reaction was carried out as a suspension; [6] I was directly solubilized in the buffer and not added as a solid.

Table 4. Time course of IMP synthesis from inosine (I).

Entry [1]	Assessed Variable(s)	PhoC-*Mm* (μg/mL (Addition Time))	PPi (mM (Addition Time))	Time (h) [2]	IMP (%) [3]
1	reference reaction	10 (t_0)	250 (t_0)	6	31
2	[Phoc-*Mm*]	20 (t_0)	250 (t_0)	4	40
3	[Phoc-*Mm*]	30 (t_0)	250 (t_0)	2	41
4	multiple additions of Phoc-*Mm* and PPi	10 (t_0), 10 (6 h)	250 (t_0)	8	37
5	multiple additions of Phoc-*Mm* and PPi	10 (t_0), 10 (6 h)	250 (t_0), 250 (6 h)	10	44
6	multiple additions of Phoc-*Mm* and PPi	20 (t_0), 20 (4 h)	250 (t_0)	4	37
7	multiple additions of Phoc-*Mm* and PPi	20 (t_0), 20 (4 h)	250 (t_0); 250 (4 h)	24	49

[1] See Figure 1 and Supplementary Materials (Paragraph S3.2) for graphs; [2] time was selected according to the highest conversion value; [3] the amount of inosine 5′-monophosphate (IMP), evaluated by HPLC, was calculated according to the following equation: IMP (%) = ([IMP]/[I + IMP]) × 100.

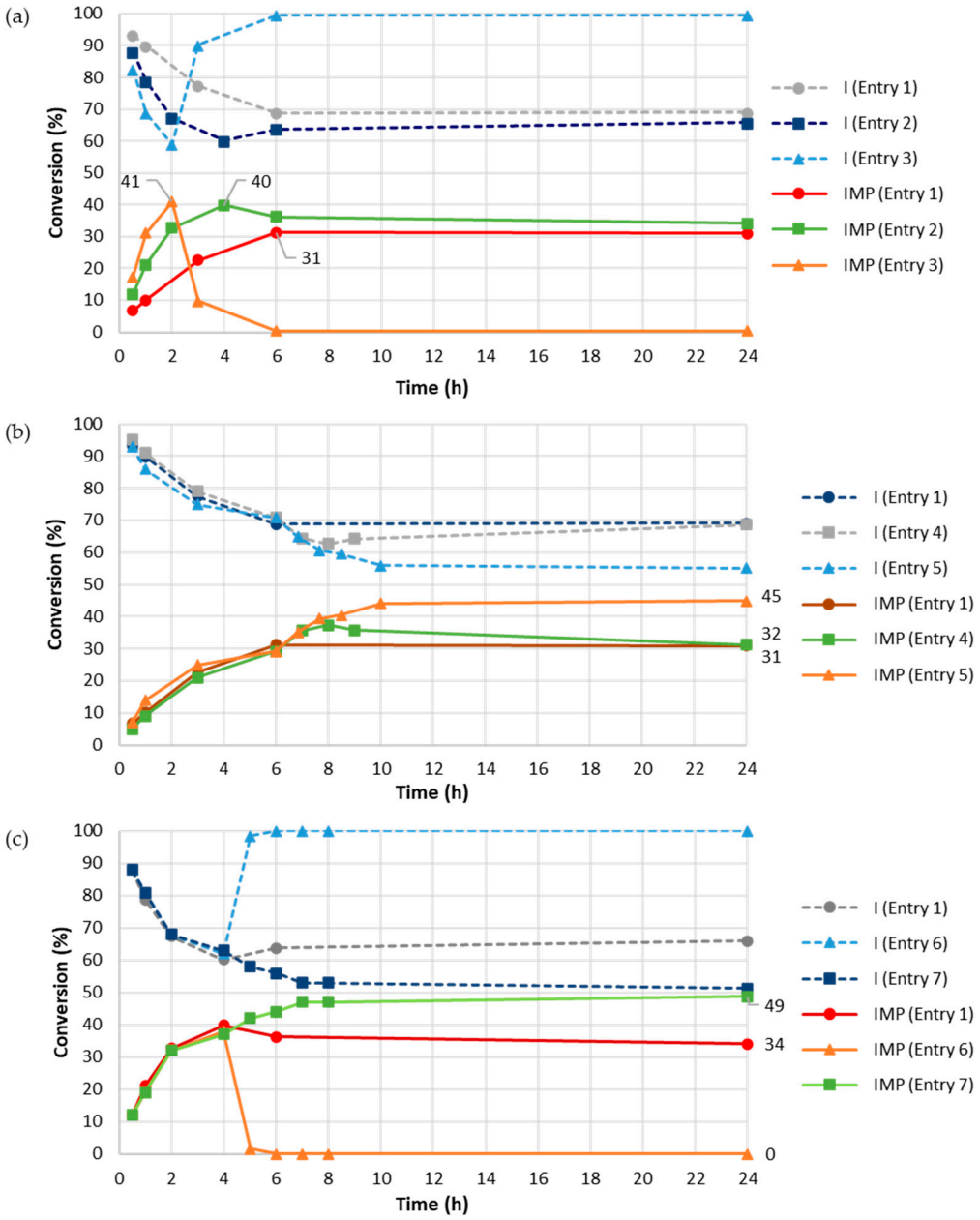


Figure 1. Conversions of I into IMP with different additions of PhoC-*Mm* and PPi. (**a**) 10, 20, and 30 μg/mL PhoC-*Mm* at t_0 (Table 4, Entries 1, 2, and 3); (**b**) 10 μg/mL PhoC-*Mm* at t_0, then 10 μg/mL PhoC-*Mm* at 6 h, with or without 250 mM PPi (Table 4, Entries 1, 4 and 5); (**c**) 20 μg/mL PhoC-*Mm* at t_0, then 20 μg/mL PhoC-*Mm* at 4 h, with or without 250 mM PPi (Table 4, Entries 1, 6 and 7).

2.5. Phosphorylation of Inosine Catalyzed by Phoc-Mm (Semi-Preparative Scale)

PhoC-*Mm* (10 or 20 μg/mL) was added to a mixture of inosine (I, 215 mg, 0.8 mmol, 80 mM) and PPi (555 mg, 2.50 mmol, 250 mM) in 100 mM NaOAc buffer (pH 5.0, 10 mL). The resulting reaction was stirred at 30 °C and consecutive additions of PhoC-*Mm* (10 or 20 μg/mL) and PPi (555 mg, 2.50 mmol, 250 mM) were made according to Figure 2. Withdrawals (20 μL) were made at appropriate times, diluted with 100 mM NaOAc buffer (pH 5.0, 180 μL), quenched with 1 M NaOH (20 μL), and analyzed by HPLC.

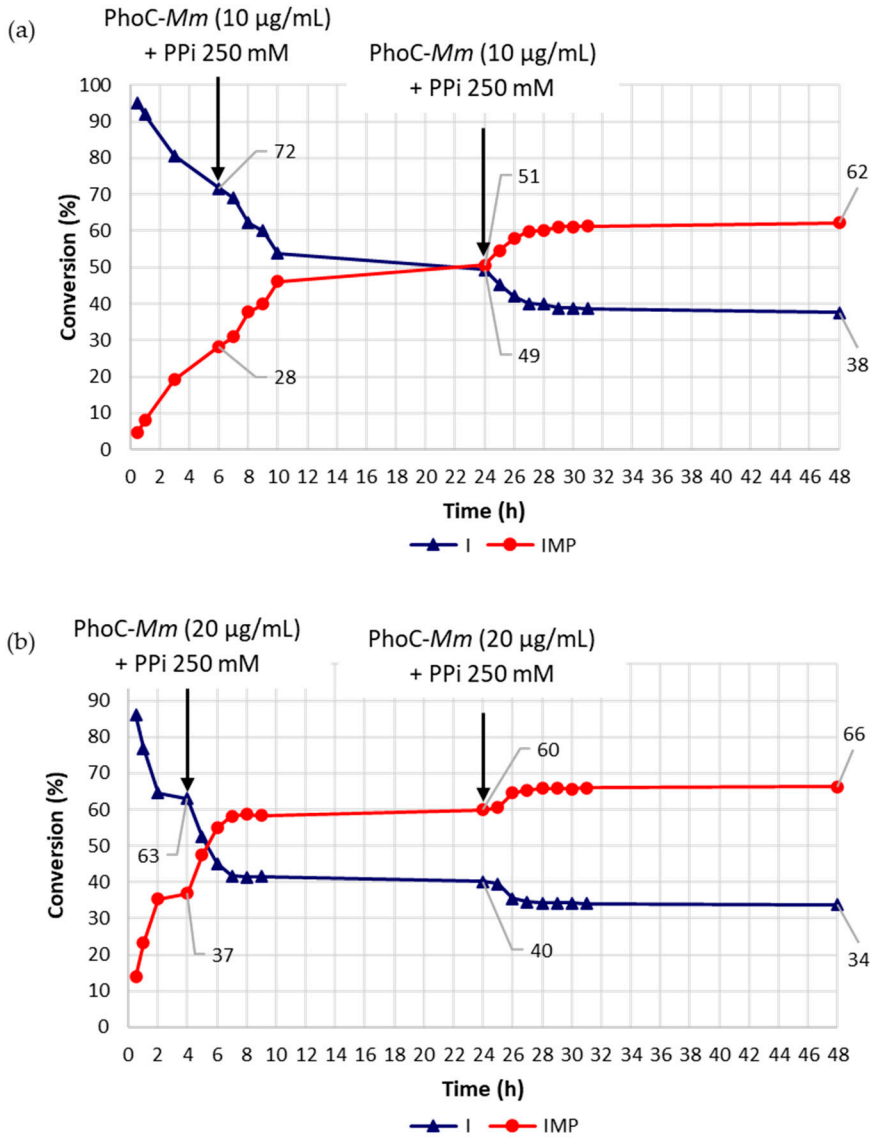


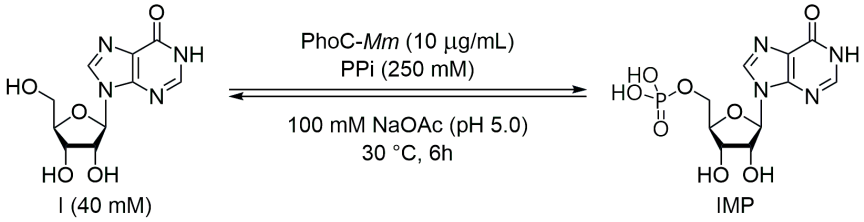
Figure 2. Semi-preparative synthesis of IMP by PhoC-*Mm*-catalyzed phosphorylation of I. (**a**) Addition of 10 µg/mL PhoC-*Mm* and 250 mM PPi at t_0, 6 h, and 24 h; (**b**) addition of 20 µg/mL PhoC-*Mm* and 250 mM PPi at t_0, 4 h, and 24 h.

After 48 h, the mixture was diluted with 100 mM NaOAc buffer (pH 5.0, 90 mL), quenched with 1 M NaOH (10 mL), freeze-dried, and the resulting crude was purified by semi-preparative HPLC to obtain IMP as a white powder (162 mg, 0.47 mmol, 58% yield for Figure 2a and 173 mg, 0.50 mmol, 62% yield for Figure 2b). Products were characterized by ^{1}H, ^{13}C, and ^{31}P NMR and MS spectroscopy (see Supplementary Materials, Paragraph S2).

3. Results and Discussion

PhoC-*Mm* was expressed in the heterologous host *E. coli* BL21(DE3) and subsequently purified to homogeneity by using His-tag affinity techniques (see Supplementary Materials, Figure S1) in high yield (76 mg/L) [26].

As a first approach, the prepared enzyme was used for the synthesis of inosine 5′-monophosphate (IMP) in experimental conditions similar to those previously described by Asano et al. [24,25], i.e., 10 µg/mL of wild-type Phoc-*Mm* and 40 mM inosine (I) in 100 mM NaOAc buffer (pH 5.0) at 30 °C. Upon monitoring the reaction progress by HPLC, a 31% conversion of I to IMP after 6 h (Scheme 2) was observed.



Scheme 2. PhoC-*Mm*-catalyzed phosphorylation of inosine (I) to inosine 5′-monophosphate (IMP) (reference reaction).

Interestingly, unlike the results reported by Asano et al. [24,25], such a value remained unchanged after 24 h (see Supplementary Materials, Table S1). This result, indicative of an optimal balance between the phosphorylating and hydrolytic activity toward inosine and IMP, respectively, could be attributed to the reported inhibitory action of inorganic phosphate [33], accumulating at a rate fast enough to prevent the hydrolysis reaction to occur after 6 h in the selected conditions.

Starting from this result and keeping the enzyme amount constant, we proceeded to optimize the reaction conditions by varying the type of buffer and amount of pyrophosphate (PPi) together with the mode/time of its addition and inosine concentration (Tables 1–3 and Supplementary Materials, Paragraph S3.1).

The data of Table 1 indicate that the substitution of NaOAc buffer with either MES (2-(*N*-morpholino)ethanesulfonic acid) or sodium citrate at the same concentrations and similar pH did not yield better results with respect to the reference reaction (Table 1, Entries 2 and 3 vs. 1), as well as lowering the concentration of PPi to 200 mM or increasing it to 400 mM (Table 2, Entries 4 and 5).

As for the mode/time of phosphate donor addition to the reaction mixture, a fed-batch approach was adopted. Using from 250 to 750 mM PPi in 3–30 h intervals (Table 2, Entries 6–8), a significant increase in the conversion up to 44% was observed (vs. 41% reported by Asano [25]) only when the highest final concentration of PPi (750 mM) was used by portionwise additions, in the presence of 80 mM of I.

The evidence that the improved conversion was related to the mode of addition/concentration of PPi was inferred by investigating the influence of the concentration of I (Table 3, Entries 9–12). The effects of the increasing inosine concentration up to 200 mM were proved to be unsuccessful in most cases, with the worst results obtained when a suspension was formed at 200 mM inosine (Table 3, Entry 10). These results can be attributed to the scarce solubility of the substrate, which could be completely dissolved in the reaction medium up to 100 mM at 30 °C.

With these results in hand, we moved to investigate the influence of single or multiple additions of increasing amounts of PhoC-*Mm* and/or PPi (Table 4), keeping all the remaining parameters constant (80 mM substrate concentration, 100 mM NaOAc (pH 5.0) buffer, 30 °C)

A comparison between the extent of phosphorylations with 10, 20, and 30 µg/mL PhoC-*Mm* at 250 mM PPi (Entries 1, 2, and 3 and Figure 1a) indicates that an increase in the enzyme amount results in the increase in the conversion of I into IMP (from 31 to 40%).

However, a 30 µg/mL PhoC-*Mm* concentration rapidly led to a decline in conversion over time, with the phosphorylated product IMP being completely hydrolyzed after only 6 h, according to the reported kinetic parameters of the wild-type enzyme ($K_{m\ inosine}$ = 122 vs. $K_{m\ IMP}$ = 0.59 mM; $v_{max\ inosine}$ = 16.24 vs. $K_{m\ IMP}$ = 76.95 µmol/mg PhoC/min) [25].

Conversely, the reaction catalyzed by 20 µg/mL PhoC-*Mm* reached a higher conversion value of IMP (40%) more rapidly (4 h) with respect to the reference reaction, despite a slight decrease to 36 and 34% after 6 and 24 h, respectively.

Then, a series of experiments with successive additions of enzyme and/or PPi were carried out (Entries 4–7), resulting in a significant improvement in IMP formation (Figure 1b,c. Among them, the best result was achieved in the case of Entry 7 (20 µg/mL PhoC-*Mm* at t_0

and 4 h, together with two concomitant additions of 250 mM PPi), in which the highest conversion value was obtained (49%). Moreover, the formed IMP was found to be stable within 24 h (see Figure 1c). The unsatisfactory results obtained with a single addition of PPi (Entry 6, 37% conversion after 4 h followed by a rapid decline) may be justified by taking into account the ratio between the biocatalyst amount and the pyrophosphate ion available in the reaction medium. Indeed, in the absence of fresh PPi as an alternative substrate after 4 h, PhoC-*Mm* would turn to processing IMP, thereby accelerating the hydrolysis reaction before being inhibited by phosphate.

Driven by this positive outcome, we scaled up the phosphorylation by applying this approach based on multiple additions of enzyme and PPi to a semi-preparative scale. Thus, 80 mM of I, dissolved in 10 mL NaOAc buffer, was converted into IMP by feeding the reaction with three fixed amounts of PhoC-*Mm* (10 or 20 μg/mL) and PPi (250 mM) at different times (t_0, 4 or 6 h, 24 h), as shown in Figure 2a,b.

As a result, the conversion values of inosine into IMP rose up to 62 and 66% by adding 10 or 20 μg/mL PhoC-*Mm*, respectively. It is worth noting that IMP was preserved from the hydrolytic activity of PhoC-*Mm* and proved to be remarkably stable within 48 h in the selected conditions. Using these optimized reaction set-ups, starting from 215 mg of inosine, 162 mg and 173 mg of pure IMP were obtained after purification by semi-preparative HPLC, corresponding to a 58 and 62% isolated yield, respectively.

In conclusion, phosphorylation of alcohols represents a key reaction both in biology and in organic synthesis due to the important role that phosphorylated molecules play in living systems and in various industrial sectors (food, pharmaceutical, cosmetic, agrochemical). In particular, purine 5′-nucleoside monophosphates, such as IMP and GMP, are of significant economic interest because they are widely used as food additives (flavor enhancers, E 631 and E 627, respectively) [6,7]. Chemical phosphorylation of nucleosides requires harsh reaction conditions, the use of toxic reagents and petroleum-based organic solvents, and the need for protection–deprotection steps, resulting frequently in poor/moderate yields associated with high energy consumption and waste disposal issues. An enzymatic approach, based on the use of phosphatases, appears to be a good alternative to introduce a phosphate group into nucleosides, overcoming the above-mentioned drawbacks and offering a much more viable and green route [17].

In this paper, the phosphotransferase activity of the wild-type phosphatase from *M. morganii* (PhoC-*Mm*) has been successfully exploited through the combined use of an inexpensive phosphate donor (pyrophosphate) and a reaction engineering approach. Such a strategy allowed the phosphorylation of inosine (I) into IMP with a conversion and isolated yield higher than those previously reported for the same biotransformation without any protein engineering or prior modification of the phosphate donor.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app14146227/s1, Paragraph S1: expression and purification of PhoC-*Mm*; Figure S1: 12% SDS-PAGE analysis of cell extracts from *E. coli* BL21 cells expressing the recombinant protein; Paragraph S2: NMR and MS spectra of inosine 5′-monophosphate (IMP); Paragraph S3: conversion curves for the phosphorylation of inosine (I) to inosine 5′-monophosphate (IMP); Paragraph S3.1: Phosphorylations on analytical scale (500 μL); Paragraph S3.2: phosphorylations on semi-preparative scale (10 mL); Paragraph S4: analytical and semi-preparative chromatograms; Table S1: Parameters optimization of the phosphorylation of I to IMP with 10 μg/mL PhoC-*Mm*.

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References

1. Bowler, M.W.; Cliff, M.J.; Waltho, J.P.; Blackburn, G.M. Why did nature select phosphate for its dominant roles in biology? *New J. Chem.* **2010**, *34*, 784–794. [CrossRef]
2. Sullenger, B.A.; Nair, S. From the RNA world to the clinic. *Science* **2016**, *352*, 1417–1420. [CrossRef] [PubMed]
3. Guo, X.; Fu, S.; Ying, J.; Zhao, Y. Prebiotic Chemistry: A review of nucleoside phosphorylation and polymerization. *Open Biol.* **2023**, *13*, 220234. [CrossRef] [PubMed]
4. Jordheim, L.P.; Durantel, D.; Zoulim, F.; Dumontet, C. Advances in the development of nucleoside and nucleotide analogues for cancer and viral diseases. *Nat. Rev. Drug Discov.* **2013**, *12*, 447–464. [CrossRef] [PubMed]
5. Pradere, U.; Garnier-Amblard, E.C.; Coats, S.J.; Amblard, F.; Schinazi, R.F. Synthesis of nucleoside phosphate and phosphonate prodrugs. *Chem. Rev.* **2014**, *114*, 9154–9218. [CrossRef] [PubMed]
6. Behrens, M.; Meyerhof, W.; Hellfritsch, C.; Hofmann, T. Sweet and umami taste: Natural products, their chemosensory targets, and beyond. *Angew. Chem. Int. Ed.* **2011**, *50*, 2220–2242. [CrossRef] [PubMed]
7. Morelli, C.F.; Pappalardo, V.; Brockhoff, A.; Pieraccini, S.; Sironi, M.; Sangiorgio, S.; Scarabattoli, L.; Speranza, G.; Rabuffetti, M. Purine 5′-ribonucleotide-L-glutamate hybrids as potential tools to investigate the mechanism of Umami taste reception. *ChemistrySelect* **2022**, *7*, e202204123. [CrossRef]
8. Roy, B.; Depaix, A.; Périgaud, C.; Peyrottes, S. Recent trends in nucleotide synthesis. *Chem. Rev.* **2016**, *116*, 7854–7897. [CrossRef]
9. Yoshikawa, M.; Kato, T.; Takenishi, T. A novel method for phosphorylation of nucleosides to 5′-nucleotides. *Tetrahedron Lett.* **1967**, *8*, 5065–5068. [CrossRef]
10. Yoshikawa, M.; Kato, T.; Takenishi, T. Studies of phosphorylation. III. Selective phosphorylation of unprotected nucleosides. *Bull. Chem. Soc. Jpn.* **1969**, *42*, 3505–3508. [CrossRef]
11. Domon, K.; Puripat, M.; Fujiyoshi, K.; Hatanaka, M.; Kawashima, S.A.; Yamatsugu, K.; Kanai, M. Catalytic chemoselective *O*-phosphorylation of alcohols. *ACS Cent. Sci.* **2020**, *6*, 283–292. [CrossRef] [PubMed]
12. Kim, H.-J.; Benner, S.A. Abiotic synthesis of nucleoside 5′-triphosphates with nickel borate and cyclic trimetaphosphate (CTMP). *Astrobiology* **2021**, *21*, 298–306. [CrossRef] [PubMed]
13. Shepard, S.M.; Jessen, H.J.; Cummins, C.C. Beyond triphosphates: Reagents and methods for chemical oligophosphorylation. *J. Am. Chem. Soc.* **2022**, *144*, 7517–7530. [CrossRef] [PubMed]
14. O'Connell, A.; Barry, A.; Burke, A.J.; Hutton, A.E.; Bell, E.L.; Green, A.P.; O'Reilly, E. Biocatalysis: Landmark discoveries and applications in chemical synthesis. *Chem. Soc. Rev.* **2024**, *53*, 2828–2850. [CrossRef] [PubMed]
15. Sheldon, R.A.; Woodley, J.M. Role of biocatalysis in sustainable chemistry. *Chem. Rev.* **2018**, *118*, 801–838. [CrossRef] [PubMed]
16. Wohlgemuth, R. The power of biocatalysts for highly selective and efficient phosphorylation reactions. *Catalysts* **2022**, *12*, 1436. [CrossRef]
17. Ubiali, D.; Speranza, G. Enzymatic phosphorylation of nucleosides. In *Enzymatic and Chemical Synthesis of Nucleic Acid Derivatives*, 1st ed.; Fernández-Lucas, J., Ed.; Wiley-VCH: Weinheim, Germany, 2019; pp. 29–42.
18. Serra, I.; Conti, S.; Piškur, J.; Clausen, A.R.; Munch-Petersen, B.; Terreni, M.; Ubiali, D. Immobilized *Drosophila melanogaster* deoxyribonucleoside kinase (*Dm*dNK) as a high performing biocatalyst for the synthesis of purine arabinonucleotides. *Adv. Synth. Catal.* **2014**, *356*, 563–570. [CrossRef]
19. Serra, I.; Ubiali, D.; Piškur, J.; Munch-Petersen, B.; Bavaro, T.; Terreni, M. Immobilization of deoxyadenosine kinase from *Dictyostelium discoideum* (*Dd*dAK) and its application in the 5′-phosphorylation of arabinosyladenine and arabinosyl-2-fluoroadenine. *ChemistrySelect* **2017**, *2*, 5403–5408. [CrossRef]
20. Robescu, M.S.; Serra, I.; Terreni, M.; Ubiali, D.; Bavaro, T. A multi-enzymatic cascade reaction for the synthesis of vidarabine 5′-monophosphate. *Catalysts* **2020**, *10*, 60. [CrossRef]

21. Fehlau, M.; Kaspar, F.; Hellendahl, K.F.; Schollmeyer, J.; Neubauer, P.; Wagner, A. Modular enzymatic cascade synthesis of nucleotides using a (d)ATP regeneration system. *Front. Bioeng. Biotechnol.* **2020**, *8*, 854.
22. Widlanski, T.S.; Taylor, W. Chemistry and enzymology of phosphatases. In *Comprehensive Natural Products Chemistry*, 1st ed.; Barton, D., Nakanishi, K., Meth-Cohn, O., Eds.; Elsevier: Amsterdam, The Netherlands, 1999; pp. 139–162.
23. Médici, R.; Garaycoechea, J.I.; Valino, A.L.; Pereira, C.A.; Lewkowicz, E.S.; Iribarren, A.M. A comparative study on phosphotransferase activity of acid phosphatases from *Raoultella planticola* and *Enterobacter aerogenes* on nucleosides, sugars, and related compounds. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 3013–3022. [CrossRef] [PubMed]
24. Asano, Y.; Mihara, Y.; Yamada, H. A New Enzymatic method of selective phosphorylation of nucleosides. *J. Mol. Catal. B Enzym.* **1999**, *6*, 271–277. [CrossRef]
25. Asano, Y.; Mihara, Y.; Yamada, H. A novel selective nucleoside phosphorylating enzyme from *Morganella morganii*. *J. Biosci. Bioeng.* **1999**, *87*, 732–738. [CrossRef]
26. Tasnádi, G.; Zechner, M.; Hall, M.; Baldenius, K.; Ditrich, K.; Faber, K. Investigation of acid phosphatase variants for the synthesis of phosphate monoesters. *Biotechnol. Bioeng.* **2017**, *114*, 2187–2195. [CrossRef]
27. Mihara, Y.; Utagawa, T.; Yamada, H.; Asano, Y. Phosphorylation of nucleosides by the mutated acid phosphatase from *Morganella morganii*. *Appl. Environ. Microbiol.* **2000**, *66*, 2811–2816. [CrossRef] [PubMed]
28. Mihara, Y.; Utagawa, T.; Yamada, H.; Asano, Y. Acid phosphatase/phosphotransferases from enteric bacteria. *J. Biosci. Bioeng.* **2001**, *92*, 50–54. [CrossRef] [PubMed]
29. Tanaka, N.; Hasan, Z.; Hartog, A.F.; van Herk, T.; Wever, R. Phosphorylation and dephosphorylation of polyhydroxy compounds by class a bacterial acid phosphatases. *Org. Biomol. Chem.* **2003**, *1*, 2833–2839. [CrossRef]
30. Mihara, Y.; Ishikawa, K.; Suzuki, E.; Asano, Y. Improving the pyrophosphate-inosine phosphotransferase activity of *Escherichia blattae* acid phosphatase by sequential site-directed mutagenesis. *Biosci. Biotechnol. Biochem.* **2004**, *68*, 1046–1050. [CrossRef]
31. Kragl, U. The role of reaction engineering in bioprocess development. *Chimia* **2020**, *74*, 378. [CrossRef]
32. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
33. VanEtten, R.L.; Waymack, P.P.; Rehkop, D.M. Transition metal ion inhibition of enzyme-catalyzed phosphate ester displacement reactions. *J. Am. Chem. Soc.* **1974**, *96*, 6782–6785. [CrossRef] [PubMed]