Preparative-Scale Enzymatic Synthesis of *rac*-Glycerol-1-phosphate from Crude Glycerol Using Acid Phosphatases and Phosphate

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Glycerol is a byproduct of biodiesel production and is generated in large amounts, which has resulted in an increased interest in its valorization. In addition to its use as an energy source directly, the chemical modification of glycerol may result in value-added derivatives. Herein, acid phosphatases employed in the synthetic mode were evaluated for the enzymatic phosphorylation of glycerol. Nonspecific acid phosphatases could tolerate glycerol concentrations up to 80 wt% and pyrophosphate concentrations up to 20 wt% and led to product titers up to 167 g L^{-1} in a kinetic approach. In the complementary thermodynamic approach, phytases were able to condense glycerol and inorganic monophosphate directly. This unexpected behavior enabled the simple and cost-effective production of *rac*-glycerol-1-phosphate from crude glycerol obtained from a biodiesel plant. A preparative-scale synthesis on a 100 mL-scale resulted in the production of 16.6 g of *rac*-glycerol-1-phosphate with a reasonable purity (≈75%).

Introduction

The worldwide production of biodiesel results in an enormous surplus of approximately 4 Mt/a of crude glycerol as a byproduct. Consequently, the valorization of glycerol has gained increasing attention.[1,2] Current applications involve the use of glycerol as an energy source[3] and as a green solvent (e.g., as a component of deep eutectic solvents).[4] The main obstacle for the use of glycerol as a platform chemical[5] is connected to the high input of energy required for its purification from the highly concentrated aqueous solutions obtained from biodiesel plants. Hence, processes that can utilize crude glycerol are economically highly desired.

An important and abundant derivative of glycerol in nature is its monophosphate ester. In eukaryotes and bacteria, *sn*-glycerol-3-phosphate (D-glycerol-1-phosphate), obtained from the stereoselective reduction of dihydroxyacetone phosphate (DHAP) during glycolysis, is a key building block of glycerophospholipids, the main components of cellular membranes. In contrast, archaea utilize the opposite enantiomer (L-glycerol-1-phosphate) to form ether-type lipids in isoprenoid sidechains.[6] In synthesis, D-glycerol-1-phosphate is employed commonly as a precursor to DHAP that serves as a nucleophile (donor) in asymmetric C–C coupling reactions catalyzed by aldolases.[7–9] As DHAP is an expensive and unstable metabolite, multi-enzymatic cascades that involve the phosphorylation of glycerol followed by oxidation to DHAP have been applied to the aldolase-mediated production of non-natural sugar derivatives.[10–12]

The phosphorylation of glycerol can be catalyzed by kinases or phosphatases that work in the synthesis mode. Kinases, such as glycerol kinase,[10,13] employ adenosine triphosphate (ATP) as the phosphate donor. This is a costly cofactor that requires recycling.[14,15] In contrast, phosphatases may be employed in transphosphorylation reactions by using inexpensive phosphate donors (P-donors), such as pyrophosphate (PP_i).[16] For instance, Pradines et al. employed calf intestine alkaline phosphatase and pyrophosphate for the phosphorylation of a number of alcohols, which included glycerol.[17] Interestingly, calf intestine phosphatase was able to utilize monophosphate and, thereby, catalyzed the direct condensation of glycerol and phosphate.[18]

We report here the exploitation of acid phosphatases for the preparative-scale enzymatic synthesis of *rac*-glycerol-1-phos-

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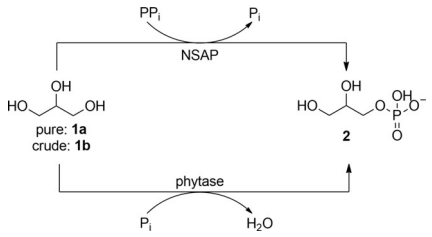

Scheme 1. Phosphorylation of glycerol (**1 a/b**) catalyzed by non-specific acid phosphatases (NSAPs) or phytase using pyrophosphate (PP_i) or monophosphate (P_i), respectively.

phate in water. In particular, our approach benefits from the bulk availability of crude glycerol and the use of cheap phosphate donors, such as PP_i and NaH_2PO_4 (Scheme 1).

Results and Discussion

A common drawback of phosphatase-catalyzed transphosphorylation reactions lies in the undesired enzyme-catalyzed hydrolysis of the newly formed products. In addition to the use of high loadings of both alcohol substrate and P-donor,[19–22] various techniques have been developed to minimize product hydrolysis, such as continuous-flow synthesis,[23,24] enzyme engineering,[25,26] and reaction engineering.[27] They could enable the mild and selective enzymatic synthesis of phosphate monoesters of functionalized alcohols and sugars.

Encouraged by these previous results, we initiated our study by testing several nonspecific acid phosphatases (NSAPs), which included PhoN-Sf from *Shigella flexneri*, PhoN-Se from *Salmonella typhimurium* LT2, PiACP from *Prevotella intermedia*, Lw from *Leptotrichia wadei*, and PhoC-Mm from *Morganella morganii*, as well as a histidine acid phosphatase and a phytase from *Aspergillus niger*[11,26] for the regioselective synthesis of glycerol-1-phosphate. High concentrations of glycerol [pure glycerol (**1 a**) and crude glycerol (**1 b**)] and PP_i as the P-donor were employed in overnight reactions at 30 °C in water. Importantly, no pH adjustment was necessary as the use of a high concentration of PP_i (used as the disodium pyrophosphate salt, $Na_2H_2P_2O_7$) resulted in a (buffered) acidic pH of approximately 3.5, a value that was beneficial in a previous study to reduce unwanted product hydrolysis in transphosphorylation reactions.[27]

Pure **1 a** and crude **1 b** (obtained from the base-catalyzed transesterification of fats with methanol) were well tolerated as substrates by most enzymes in up to 80 wt% by using 5 wt% PP_i (Figures S1 and S2). The best results were obtained by using PhoC-Mm with 60 wt% **1 a** over 24 h, which led to a 100% PP_i consumption, a 46 g L^{-1} product titer, and a 0.88 product/P_i ratio (Figure S1), which corresponds to 94% transphosphorylation efficiency.[28] The corresponding values obtained with 70 wt% **1 b** were 94%, 41 g L^{-1}, and 0.91, respectively (Figure S2), which indicates a highly effective transphosphorylation of crude glycerol (96% efficiency). Although 10 wt% PP_i was well tolerated by PhoN-Sf, PhoN-Se, and PiACP and yielded high product titers and higher product/P_i ratios (Figure S3), strongly diminished activities were obtained at 20 wt% PP_i concentration (maximum 20% PP_i consumption within 2 d; data not shown). An increase of the amount of water, which was achieved by lowering the concentration of **1 a** and PP_i (55 and 18 wt%, respectively), had a beneficial effect on enzyme activity and allowed access to higher product titers (up to 101 g L^{-1}), although PP_i consumptions were incomplete (Figure S3). A similar trend was observed with **1 b**, and surprisingly, product titers (up to 118 g L^{-1}) and PP_i consumption levels were generally superior (Figure S4). The highest product/P_i ratio was achieved with PhoC-Mm (up to 0.88), which indicates the most effective transphosphorylation, however, both product titer and PP_i consumption were lower than those obtained with PiACP and PhoN-Sf. A product/P_i ratio of approximately 0.70 could be maintained with PiACP and PhoN-Sf regardless of the enzyme amount or reaction time. Acid phosphatase Lw showed a performance very similar to that of PhoC-Mm, whereas PhoN-Se was the most active enzyme, however, its strong hydrolytic activity resulted in low product/P_i ratios. Overall, PiACP could deliver 101 g L^{-1} of **2** within 24 h from **1 a** and up to 121 g L^{-1} after 4 d (Table 1, entries 1 and 2). With **1 b**, PhoN-Sf afforded 118 g L^{-1} of **2** in 24 h and reached an impressive level of 167 g L^{-1} within 4 d (entries 4 and 5). The use of double the amount of enzyme (8 U mL^{-1}) allowed a significantly higher PP_i consumption (82% with both enzymes) and product titer (133 g L^{-1} for PiACP with **1 a** and 151 g L^{-1} for PhoN-Sf with **1 b**) already after 24 h (entries 3 and 6).

The use of phytase from *Aspergillus niger* (phytase cc; see Supporting Information), which has been reported to convert **1 a** non-stereoselectively to *rac*-glycerol-1-phosphate,[11] resulted in a nearly full consumption of PP_i in 24 h and a low product/P_i ratio (up to ≈0.2) even at 20 wt% PP_i with **1 a** (Table S2). An increased concentration of **1 a** allowed a slightly better product/P_i ratio (≈0.3; Table S2, entries 8 and 9). In the case that **1 b** was used, a similar trend was observed (Table S3). Interestingly, product formation took place even upon full PP_i consumption (Table S2, entries 2–4 and Table S3, entries 2–7). A detailed time study indicated that PP_i was hydrolyzed fully in less than 10 min regardless of the concentration used (5–

Table 1. Selected results (highest product titers) obtained with class A acid phosphatases in transphosphorylation using pyrophosphate as donor.

Entry	1	Enzyme	Enzyme amt[a] [μg mL^{-1}]	Reaction time [h]	$Cons._{PPi}$ [%]	2[b] [g L^{-1}]	[Product]/[P_i]
1	**1 a**	PiACP	43 (4)	24	59	101	0.70
2	**1 a**	PiACP	43 (4)	96	71	121	0.69
3	**1 a**	PiACP	86 (8)	24	82	133	0.67
4	**1 b**	PhoN-Sf	72 (4)	24	68	118	0.75
5	**1 b**	PhoN-Sf	72 (4)	96	97	167	0.73
6	**1 b**	PhoN-Sf	144 (8)	24	82	151	0.74

[a] Enzyme amount expressed in U mL^{-1} in parentheses; [b] product is racemic. Reaction conditions: 55 wt% **1 a/b**, 18 wt% PP_i, 1 mL reaction volume, pH≈3.5, 1% DMSO as internal standard, 30 °C, 1400 rpm shaking. $Cons._{PPi}$ = consumption of PP_i. Product titer was calculated from the molecular weight of the monosodium phosphate ester salt.

20 wt%) and that product formation only happened afterwards, which means that the reaction took place in the presence of monophosphate only (Figure S5). Inspired by this observation, we studied different monophosphate salts (NaH_2PO_4 and KH_2PO_4) as the P-donor by using 64 wt% glycerol and 20 wt% P_i. Similar to the use of PP_i, the use of monobasic phosphate salts allowed a simple reaction set-up without pH adjustment (final pH ≈ 3.5). Two types of phytase were tested at three different concentrations, Natuphos and phytase cc (Natuphos E). Both enzymes are produced by BASF on a >1000 t/a scale as an additive for animal feed to improve phosphate availability. Although Natuphos is a 3-phytase from a fungal origin (*Aspergillus*),[29] phytase cc is a 6-phytase,[30] which is a hybrid of various bacterial phytases developed for increased stability towards proteolytic digestion and elevated temperatures. Interestingly, KH_2PO_4 was a poor donor compared to the sodium salt (**2** was obtained at a maximum of 17 g L^{-1}; data not shown); the latter delivered in contrast conversion levels up to approximately 20% and product titers up to 75 g L^{-1} in 29 h and 24% and 104 g L^{-1}, respectively, after 4 d (Figure S6, Table 2 entries 1, 2, 4, and 5). The main difference between the two types of phytase lies in the initial velocity of the reaction (Figures S6 and S7). The conversion using Natuphos reached a plateau faster than that with phytase cc, however, maximum product levels were comparable over longer incubation times, and 20 U mL^{-1} enzyme appeared optimal for increased product formation. Given the successful use of inorganic monophosphate as the P-donor by phytase, it appears that the enzyme catalyzes a reversed hydrolytic reaction (Scheme 1) instead of transphosphorylation (according to the known mechanism[16]). The reaction likely reaches an equilibrium that depends on the reaction parameters (most notably, substrate and phosphate concentration, temperature, and water content). In particular, **1b** delivered slightly lower conversions (up to 21%) and product concentrations (up to 75 g L^{-1}) compared to **1a**, which presumably is the result of the presence of residual H_2O (≈10% content) in **1b** (Figure S7, Table 1 entries 7, 8, 12, and 13), which impacts the reaction equilibrium. Interestingly, class A acid phosphatases PhoN-Sf, PhoN-Se, PiACP, and Lw tested under similar conditions also showed product formation, although to a lower extent compared to the two phytases (maximum 15 g L^{-1} product concentration within 2 d; Table S4). Furthermore, these enzymes preferred KH_2PO_4 over NaH_2PO_4. This observation suggests that the ability of acid phosphatases to reverse the hydrolysis of phosphate esters is a general property, which has not been considered so far. However, the extent of ester formation with an inorganic monophosphate as a donor is highly enzyme dependent. The good performance of phytases under these conditions lies in (so far) unknown mechanistic details and provides a catalytic system with a high atom economy of interest for synthetic applications as no byproduct is generated, except water. The high space–time yield achieved (up to 20 g L^{-1} d^{-1}) appears particularly attractive for the synthesis of glycerol monophosphate under industrial conditions.

Next, the effect of temperature on the equilibrium of the reaction between glycerol and monophosphate was studied. Both types of phytase as well as **1a** and **1b** were subjected to screening conditions (Figures S8 and S9, respectively; results obtained at 30 °C are given for comparison). Both enzymes were active at 60 °C, although only phytase cc is considered thermostable. The high concentration of glycerol, a known enzyme-stabilizing agent,[31] may have a beneficial effect on the thermal stability of Natuphos. The elevated temperature significantly enhanced the rate of product formation and thereby improved maximum product titers by approximately 30–39% (e.g., from 75 to 104 g L^{-1} with **1a** and 67 to 87 g L^{-1} with **1b** in 24 h, at 30 and 60 °C, respectively; Table 2, entries 3, 6, 9, and 14). As noted earlier, lower conversions (maximum ≈22%) and product concentrations (maximum 87 g L^{-1}) were obtained with **1b**. The difference in the initial activity of the enzymes remained unchanged at 60 °C, and conversion with Natuphos again reached a plateau faster than that with phytase cc.

Finally, **1a** and **1b** were mixed with NaH_2PO_4 without added H_2O, followed by the addition of phytases. The reaction was catalyzed successfully by phytase cc at 60 °C and reached approximately 28% conversion with **1b** within 24 h (vs. ≈22% in the presence of added H_2O) to attain approximately 33% after 48 h (Table 2, entries 10 and 11). Natuphos was inactive in the absence of added water. Neither of the enzymes was active in **1a**.

The activity of phytase cc in **1b** without added water simplifies the reaction set-up significantly and also allows the recycling of unreacted P_i, which contributes positively to the sustainability and the commercial attractiveness of the process overall. Therefore, we investigated the scalability of this system along with a suitable isolation protocol for the prod-

Table 2. Selected results obtained with two phytases in phosphorylation reactions with monophosphate salt as the donor.

Entry	1	Enzyme	Enzyme amt[a] [μg mL^{-1}]	T [°C]	t [h]	Conv. [%]	2 [g L^{-1}]	[Product]/[P_i]
1	**1a**	phytase cc	201 (30)	30	29	15	56	0.21
2	**1a**	phytase cc	201 (30)	30	101	22	80	0.24
3	**1a**	phytase cc	201 (30)	60	24	25	101	0.33
4	**1a**	Natuphos	588 (30)	30	29	20	75	0.29
5	**1a**	Natuphos	588 (30)	30	101	24	86	0.26
6	**1a**	Natuphos	588 (30)	60	24	26	104	0.34
7	**1b**	phytase cc	201 (30)	30	29	14	50	0.17
8	**1b**	phytase cc	201 (30)	30	101	18	67	0.20
9	**1a**	phytase cc	201 (30)	60	24	22	85	0.28
10[b]	**1a**	phytase cc	201 (30)	60	24	28	139	0.39
11[b]	**1a**	phytase cc	201 (30)	60	48	33	165	0.50
12	**1b**	Natuphos	588 (30)	30	29	18	67	0.23
13	**1b**	Natuphos	588 (30)	30	101	21	75	0.23
14	**1a**	Natuphos	588 (30)	60	24	22	87	0.29

[a] Enzyme amount expressed in U mL^{-1} in brackets; [b] reaction contained 1 mL **1b** and 350 mg NaH_2PO_4. Reaction conditions: 64 wt% **1a/b**, 20 wt% NaH_2PO_4, 1 mL reaction volume, pH ≈ 3.5, 1% DMSO as internal standard, 1400 rpm shaking. Conversions with respect to the limiting reactant (NaH_2PO_4). Product titer was calculated from the molecular weight of the monosodium phosphate ester salt.

uct. A phosphorylation reaction on a preparative scale (i.e., 100 mL **1b** and 40.0 g NaH_2PO_4) was performed at 60 °C and led to 33% conversion within 19 h. Unreacted P_i was first removed by precipitation as a magnesium ammonium salt (struvite).[32,33] Product **2** was finally isolated as the magnesium salt in 26% yield (16.6 g) and approximately 75% purity (measured by NMR spectroscopy), which is an acceptable value given the simplicity of the overall set-up and the crude nature of the starting material. The detected impurities consisted of approximately 5% P_i, 10% glycerol-2-phosphate, and 10% glycerol. Finally, an enzymatic assay coupled with ^{31}P NMR spectroscopy indicated that **2** obtained from the preparative-scale synthesis was racemic (Figures S14 and S15).

Conclusions

Several acid phosphatases were investigated at high substrate loadings for the catalyzed (trans)phosphorylation of glycerol to produce *rac*-glycerol-1-phosphate. The effect of the type of phosphate donor (pyrophosphate or monophosphate salt) and of the type (crude or pure) and concentration of glycerol as well as the reaction temperature were studied. Nonspecific acid phosphatases retained their activity in the presence of 18 wt% pyrophosphate as the phosphate donor and 55 wt% glycerol to result in the production of up to 167 g L^{-1} glycerol-1-phosphate. In contrast, phytases could utilize 20 wt% NaH_2PO_4 as the monophosphate salt for the direct condensation in 64 wt% glycerol to furnish 104 g L^{-1} product. In comparison, calf intestine alkaline phosphatase, which also delivers approximately 10% of glycerol-2-phosphate in the phosphorylation of glycerol,[17] could deliver 71 g L^{-1} product at best,[18] whereas the product titer with nonspecific acid phosphatases reached only approximately 17 g L^{-1} from pyrophosphate under standard conditions.[26,27] Importantly, several enzymes could operate efficiently in crude glycerol obtained from biodiesel production. Moreover, phytase cc was able to work under neat conditions, which enabled the development of a preparative-scale synthesis on a 100 mL scale and the isolation of *rac*-glycerol-1-phosphate by fractional crystallization as a magnesium salt in 26% yield (16.6 g).

Experimental Section

Standard conditions for small-scale transformations with added water

A solution was prepared that contained **1a** or **1b** and P-donor ($Na_2H_2P_2O_7$, NaH_2PO_4, or KH_2PO_4) in H_2O at the concentrations [wt%] indicated in the footnotes of tables and captions of figures with 1 vol% DMSO as an internal standard. The P-donors were used as fine powders. The resulting solution had a pH of ≈3.5; no pH adjustment was applied. The suspension was shaken thoroughly, and 1 mL was transferred into a 1.5 mL screw-capped glass vial followed by the addition of the corresponding amount of enzyme (27–588 μg mL^{-1}; Table S1). The mixture was shaken vigorously in an Eppendorf thermoshaker at 1400 rpm at 30 or 60 °C for 1–4 d. Samples (25 μL) were diluted with aq. H_2SO_4 (475 μL of 8 mM for NSAPs or 140 mM for phytases) and analyzed by HPLC with refractive index (RI) detection. Experiments were performed in duplicate. Product titer [g L^{-1}] was calculated from the molecular weight of the monosodium phosphate ester salt.

Standard conditions for small-scale transformations without added water

To a 1.5 mL screw-capped glass vial were added NaH_2PO_4 (350 mg) and **1a** or **1b** (1 mL) followed by phytase cc (201 μg mL^{-1}) or Natuphos (588 μg mL^{-1}). The mixture was shaken vigorously by using an Eppendorf thermoshaker at 1400 rpm at 60 °C for 2 d. Samples (25 μL) were diluted with aq. H_2SO_4 (475 μL, 140 mM) and analyzed by HPLC–RI. Experiments were performed in duplicate.

Preparative-scale phosphorylation of 1b

Compound **1b** (100 mL), NaH_2PO_4 (40.0 g, 333 mmol), and phytase cc (100 mg) were stirred at 60 °C in a 250 mL round-bottomed flask (Figure S10). The reaction was quenched after 19 h by adding the mixture to NaOH (500 mL, 0.5 M). The resulting solution (≈620 mL) had a pH of ≈7.5. Then, DMSO (final 1 vol%) was added, and the concentrations of unreacted P_i and **2** were measured by using HPLC–RI, which indicated 168 mM of product and 345 mM of P_i, which corresponds to ≈33% conversion with respect to P_i. Next, NH_4Cl (1 equiv., 17.8 g, 333 mmol) was added, and the pH was adjusted to 7.5–8.0. Then, $MgCl_2{\cdot}6H_2O$ (1 equiv., 67.8 g, 333 mmol) was added portionwise, and the pH was kept between 7.0 and 8.0 by adding NaOH (10 M). The resulting suspension was stirred for 5 min followed by filtration (glass frit porosity 3). The solid residue was washed with H_2O (≈20 mL). Finally, the pH of the filtrate was adjusted to ≈8.0, and EtOH (4 volumes) was added to the mixture. The product was allowed to precipitate at 4 °C overnight. Product stability during the work-up procedure was checked by taking a sample before treatment with EtOH. The precipitation resulted in a sticky solid, which was impossible to filter. Instead, the supernatant was decanted, and the product was dissolved in water followed by lyophilization overnight. This step gave a light brownish powder (16.6 g, yield 26%). The product was analyzed by NMR spectroscopy, which showed ≈75% (molar) purity (Figures S11–S13). The impurities consisted of ≈5% P_i, 10% glycerol-2-phosphate, 10% glycerol, and traces of EtOH.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: alcohols · biocatalysis · enzyme catalysis · phosphorylation · synthesis design

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