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Optimization of oxidative bioconversions catalyzed by phenylacetone monooxygenase from *Thermobifida fusca*

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ABSTRACT

By choosing properly the nature of the reaction medium and its ionic strength, biocatalytic properties of isolated phenylacetone monooxygenase from *Thermobifida fusca* can be improved, achieving the best results when working in Tris or phosphate buffers presenting moderate ionic strengths. The use of different enzymatic cofactor regenerating systems has been studied, resulting in the highest activities by using glucose or glucose-6-phosphate dehydrogenase. The cofactor concentration, key parameter when oxidizing with isolated Baeyer–Villiger monooxygenases, was optimized, being demonstrated that PAMO can perform its biocatalytic activity with the highest TTNs with low requirement of nicotinamide cofactor (2 μM).

1. Introduction

An interesting set of enzymes to apply for oxidative biocatalysis are the Baeyer–Villiger monooxygenases (BVMOs). These redox enzymes have emerged as useful tools for catalyzing chemo-, regio- and/or enantioselective oxidations difficult to achieve by the conventional chemical methods [1–4].

One particularly interesting BVMO is phenylacetone monooxygenase from *Thermobifida fusca* (PAMO, EC 1.14.13.92), which was recently cloned, overexpressed and structurally characterized [5,6]. This thermostable flavoprotein catalyzes the Baeyer–Villiger reaction of carbonylic compounds, as well as heteroatom oxidations. In this regard, PAMO was demonstrated to be a very effective and selective biocatalyst for the kinetic resolution of racemic benzylketones in order to obtain the corresponding (*S*)-esters and (*R*)-ketones [7,8]. Moreover, PAMO has been employed as enantioselective sulfoxidation biocatalyst [9].

Biocatalysts are sensitive to the employed reaction conditions. Thus, the manipulation of the physical environment of proteins in order to control the enzymatic activity and/or selectivity has become an attractive target. Besides pH and temperature, the effect of which on PAMO activity and selectivity has been previously shown [8,10], there are other factors that may alter the enzyme properties, such as the nature or the ionic strength of the reaction medium [11–14] in which oxidative processes are developed.

The use of isolated enzymes offers some advantages with respect to whole cells systems, since this practise steers clear of the enzymatic co-metabolism of substrates, diminishes the substrate/product toxicity and avoids the problems for transporting these compounds in and out of the cells [15]. However, isolated oxidoreductases require the presence of cofactors as a source of electrons to perform their catalytic activity. Due to the prohibitive cost associated with stoichiometric use of nicotinamide cofactors and the problems derived from inhibitory effects on the enzymes, an effective method for recycling NAD(P)H is required. While chemical, electrochemical or photochemical approaches have been proposed for that purpose [16–19], the enzymatic regeneration systems are still the method of choice when performing BVMO-catalyzed reactions. These enzymatic methods reduce technological complications and are highly selective yielding only the 1,4-NAD(P)H isomer. Dehydrogenases are often used for oxidizing a sacrificial co-substrate in the reaction medium in order to regenerate NAD(P)H. Although other types of enzymes have been recently described, such hydrogenases [20], their application is not widespread. In case of reactions catalyzed by isolated NADPH-dependent BVMOs, glucose-6-phosphate dehydrogenase (G6PDH) from *Leuconostoc mesenteroides* [21] has been widely employed. Nevertheless, there are other alternatives, such as glucose dehydrogenase (GDH) from *Bacillus* sp. [22], alcohol dehydrogenase from *Thermoanaerobium brockii* (TBADH) [23] and phosphite dehydrogenase (PTDH) from *Pseudomonas stutzeri* [24,25]. The latter

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enzyme has been recently fused to different BVMOs for obtaining self-sufficient redox biocatalysts, the so-called CRE-BVMOs (CRE: *Coenzyme Regeneration Enzyme*) [26]. A second generation of these bifunctional enzymes (CRE2-BVMO) includes a polyhistidine tag and a thermostable mutant of PTDH [27], for optimizing the purification process and the efficiency of the biocatalytic system [28].

For the large-scale application of cofactor-dependent enzymatic synthesis, not only a proper regeneration system is required, but also the optimization of the cofactor amount is essential. The utilization of a cofactor can be expressed by its total turnover number (TTN), moles of product formed per mol of cofactor used for the reaction, and by the turnover frequency (TOF), referred as the TTN per unit of time. Higher TTN and TOF values ensure more effective biotransformations. It has been established that TTNs higher than 10^3 may be sufficient to make a process economically viable [29].

In this study the influence of the reaction medium and the type of cofactor regenerating system on the biocatalytic properties of isolated PAMO has been analyzed. The optimization of Baeyer–Villiger oxidations and sulfoxidations catalyzed by PAMO has been carried out by comparing different enzymatic cofactor regeneration systems and by optimizing the amount of NADPH required during the enzymatic process in order to ensure more effective oxidative processes. This has revealed that for an optimal PAMO-based biocatalytic process, it is crucial to select a proper reaction medium and cofactor regeneration system.

2. Experimental

2.1. General materials and methods

Recombinant histidine-tagged phenylacetone monooxygenase (PAMO) [5], phosphite dehydrogenase (PTDH E175A/A176R) [25] and bifunctional CRE2-PAMO fusion protein [28] were purified as previously described. One unit of PAMO was defined as the amount of enzyme that oxidizes 1.0 μmol of phenylacetone to benzyl acetate per minute at pH 8.0 and 30 °C in the presence of NADPH. D-Glucose-6-phosphate dehydrogenase (G6PDH 640 U/mg) from *L. mesenteroides* and alcohol dehydrogenase (TBADH 5.28 U/mg) from *T. brockii* were products from Sigma–Aldrich. D-Glucose dehydrogenase 002 (GDH 30 U/mg) was purchased from Codexis, as well as the sodium salts of the nicotinamide coenzymes $NAD(P)^+$ and NAD(P)H (purity ≥ 99%).

Phenylacetone (**2a)** was purchased from Merck. Benzyl acetate (**2b)**, thioanisole (**4a**), methyl phenyl sulfoxide (**4b**), D-glucose, D-glucose-6-phosphate and sodium phosphite were supplied by Sigma–Aldrich. (±)-3-Phenylbutan-2-one [(±)-**1a**] was prepared according to the literature, using methyl iodide and NaOH in a biphasic medium (46% yield) [30]. 3-Methyl-4-phenylbutan-2-one [(±)-**3a**] were obtained according to the literature with 30% yield, by Heck arylation of 3-methyl-3-buten-2-ol, using iodobenzene in presence of palladium chloride, tetra-*n*-butylammonium bromide and $NaHCO_3$ [31]. 3-Methyl-3-buten-2-ol was prepared by addition of methyl magnesium iodide to a methacrolein solution in diethyl ether. Esters (±)-**1b** and (±)-**3b** were prepared by chemical acylation of commercial (±)-1-phenylethanol or (±)-1-phenyl-2-propanol, respectively (yields higher than 80%). All others solvents and reagents used were of highest quality grade available. Compounds **1a** [32], **3a** [33], **1b** [34] and **3b** [35] exhibit physical and spectral properties in accord with those reported.

Absolute configuration of (*S*)-**1b** and (*S*)-**3b** was established by comparison with an authentic sample prepared from chemical acylation of the corresponding commercial chiral alcohol. Absolute configuration of sulfoxide (*S*)-**4b** was established by comparison of the HPLC chromatograms with the patterns described in previous experiments for the known configurations.

Flash chromatography was performed using Merck silica gel 60 (230–400 mesh). ^{1}H NMR, ^{13}C NMR and DEPT spectra were recorded, with TMS (tetramethylsilane) as the internal standard, on a Bruker AC-300-DPX (^{1}H: 300.13 MHz and ^{13}C: 75.4 MHz) spectrometer. ESI^+ using a HP1100 chromatograph mass detector or EI with a Finigan MAT 95 spectrometer was used to record mass spectra. GC analysis were performed on a Hewlett Packard 6890 Series II chromatograph equipped with HP-1 cross-linked methyl siloxane column (30 m × 0.32 mm × 0.25 μm, 1.0 bar N_2) for achiral analyses and Restek RtβDEXse (30 m × 0.25 mm × 0.25 μm, 1.0 bar N_2) for chiral determinations. For all the analyses, the injector temperature was 225 °C and the FID temperature was 250 °C. The following temperature programs were employed: (1) 70 °C (5 min), 1 °C min^{-1} 120 °C, t_R (*R*)-**1a**: 44.3 min; t_R (*S*)-**1a**: 46.4 min; t_R (*S*)-**1b**: 42.9 min; t_R (*R*)-**1b**: 50.5 min; (2) 70 °C (7 min), 10 °C min^{-1} 90 °C, t_R **2a**: 6.0 min; t_R **2b**: 7.7 min; and (3) 90 °C (30 min), 5 °C min^{-1} 120 °C, t_R (*R*)-**3a**: 46.9 min; t_R (*S*)-**3a**: 48.5 min; t_R (*S*)-**3b**: 42.7 min; t_R (*R*)-**3b**: 45.8 min. HPLC analyses were developed with a Hewlett Packard 1100 LC liquid chromatograph. The following conditions were used for the determination of the conversion and the enantiomeric excess of **4b**: Chiralcel OD column (0.46 cm × 25 cm), isocratic eluent: *n*-hexane/^{i}PrOH (90:10), 20 °C, flow 1 mL min^{-1}. t_R **4a** 5.3 min; t_R (*R*)-**4b** 11.2 min; t_R (*S*)-**4b** 14.2 min.

2.2. General procedure for the enzymatic oxidations catalyzed by isolated PAMO

Ketones (±)-**1a**, **2a**, (±)-**3a** or sulfide **4a** were dissolved in the corresponding reaction media (different pHs and concentrations, 1.0 mL), containing the cosubstrate (20 mM or 5–70% (v/v) when ^{i}PrOH was employed), the cofactor regeneration enzyme (5.0 units), NADPH (0.2 mM) and PAMO (1 unit). The mixture was shaken at 250 r.p.m. and the selected temperature in a rotatory shaker for the times indicated. The reaction was then stopped, worked up by extraction with EtOAc (3 × 0.5 mL), dried over Na_2SO_4 and analyzed directly by GC or HPLC to determine the conversion and enantiomeric excess in case of compounds (*R*)-**1a**, (*S*)-**1b** and (*S*)-**3a**, (*R*)-**3b**.

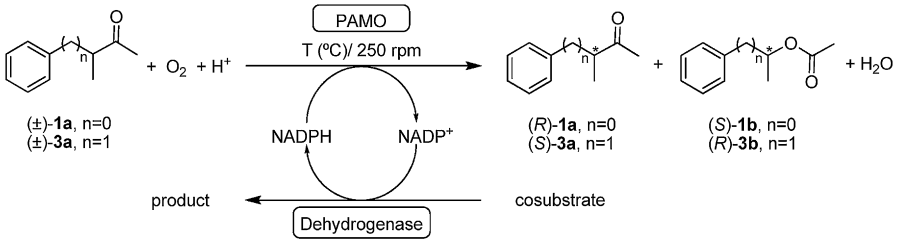
2.3. Baeyer–Villiger oxidations or sulfoxidations catalyzed by the fusion protein CRE2-PAMO

Substrates (±)-**1a**, **2a**, (±)-**3a** or **4a** were dissolved in 50 mM Tris/HCl (1.0 mL), containing sodium phosphite (10 mM), NADPH (0.2 mM) and the fusion protein CRE2-PAMO (4.0 U). The mixture was shaken at 250 r.p.m. and the selected temperature in a rotatory shaker for the times indicated. The reaction was then stopped, worked up by extraction with EtOAc (3 × 0.5 mL), dried over Na_2SO_4 and analyzed directly by GC or HPLC to determine the conversion and enantiomeric excesses in case of compounds (*R*)-**1a**, (*S*)-**1b** and (*S*)-**3a**, (*R*)-**3b**.

3. Results and discussion

3.1. PAMO-catalyzed Baeyer–Villiger oxidation of racemic 3-phenylbutan-2-one in different reaction media

The effect of the reaction media on the biocatalytic properties of PAMO when oxidizing (±)-3-phenylbutan-2-one [(±)-**1a**] was analyzed using the coupled enzymatic system glucose-6-phosphate/glucose-6-phosphate dehydrogenase to regenerate the NADPH consumed in the enzymatic oxidation (Scheme 1). All the buffer solutions were of the same concentration (50 mM) and pH (pH 9.0), with the exception of sodium tetraborate (so-called Borax) and sodium bicarbonate ($NaHCO_3$) solutions (pH 9.5). Oxidation of (±)-**1a** in Tris–HCl or phosphate buffer led to a similar



Scheme 1. General scheme for PAMO-catalyzed biooxidation of racemic benzylketones in aqueous reaction media.

Table 1
Effect of the reaction medium on the activity and selectivity of PAMO when catalyzing the Baeyer–Villiger oxidation of ketone (±)-**1a**.[a]

Reaction medium (50 mM)	*t* (h)	*ee* (%)[b] **1a**	*ee* (%)[b] **1b**	*c* (%)[c]	*E*[d]
Tris	1	50	97	34	120
Phosphate	1	51	97	34	126
Imidazole	1	63	96	40	94
HEPES	0.5	71	94	43	68
$NaHCO_3$	0.5	83	87	49	39
Borax	48	n.d.	n.d.	≤3	n.d.
Glycine	48	33	97	25	91
Triethanolamine	48	65	97	40	129

n.d., Not determined.
[a] Reaction conditions: 20 °C and pH 9.0, except for Borax and $NaHCO_3$ (pH 9.5).
[b] Determined by GC.
[c] Conversion, $c = ee_s/(ee_s + ee_p)$.
[d] Enantiomeric ratio, $E = \ln\{(1-ee_s)/[1+(ee_s/ee_p)]\}/\ln\{(1+ee_s)/[1+(ee_s/ee_p)]\}$ (see Ref. [36]).

conversion (c = 34% after 1 h, Table 1) and enantioselectivity [36] ($E \sim 120$). Higher enzymatic activities were found when employing an imidazole–HCl buffer (c = 40% after 1 h), but with a slight loss in PAMO selectivity (E = 94). This decrease in the enzymatic selectivity was higher when oxidizing (±)-**1a** in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). The employment of this buffer led to relative fast formation of (*S*)-**1b** after half an hour but with a relative low enantioselectivity. In a similar way, the use of a sodium carbonate buffer led to a fast oxidation, while an important decrease in the enantioselectivity was achieved. No significant oxidation was observed when the Baeyer–Villiger reaction was performed in Borax after 48 h, while the enzymatic resolution of (±)-**1a** was extremely slow in glycine–NaOH and triethanolamine–HCl. Thus, only 25% and 40% of the final ester was recovered after 48 h, in a process with E = 91 and E = 129, respectively. These data show that by choosing properly the reaction medium, it is possible to perform the kinetic resolution of (±)-**1a** with high activities and selectivities.

3.2. Effect of ionic strength in the enzymatic Baeyer–Villiger oxidation of (±)-3-phenylbutan-2-one catalyzed by isolated PAMO

The concentration of salts in the medium affects the electrostatic and non-polar interactions of proteins. Concentrations below 0.1 M alter the surface charge of the enzyme, whereas higher salt concentrations, not only neutralize the electrostatic forces of the protein surface, but also affect the three dimensional structure [37]. This suggests that the adjustment of ionic strength of the reaction medium can alter the enzyme stability, activity and selectivity. Baeyer–Villiger oxidation of (±)-**1a** has been performed using Tris–HCl at different concentrations. In order to compare the enzymatic activity, the space time yield, defined as mmol of ketone (±)-**1a** consumed per L of solution h^{-1}, was determined for each buffer concentration. As can be seen in Fig. 1, this parameter presents a low value when working at diluted Tris concentrations (0.75 mmol L^{-1} h^{-1} in 10 mM Tris–HCl). A maximum value of space time yield was observed at 100 mM (12.25 mmol L^{-1} h^{-1}). More concentrated buffer led to a slight decrease in the enzymatic activity, probably due to changes in the three-dimensional protein structure [37]. Nevertheless, PAMO can be used in hypersaline media since the enzyme still remains active in 2.0 M Tris–HCl, catalyzing the oxidation of 4.40 mmol of (±)-**1a** per L in an hour. This result highlights the ability of this BVMO for performing well in harsh reaction conditions (35% of (*S*)-**1b** after 1 h). Concerning the enantioselectivity, low buffer concentrations afforded high enantioselectivities, while the best *E*-values were obtained in 50–100 mM Tris–HCl (E > 100). Higher concentrations produced a slight loss in enzymatic selectivity (E = 75 at 1.5 M Tris–HCl). A moderate selectivity (E = 43) was even observed in Tris–HCl 2.0 M. The fact that PAMO is able to catalyze oxidations with good biocatalytic properties at high ionic strengths motivated us to study its applicability in mixtures aqueous buffer/ionic liquids [38] and can serve to extend its application/exploitation/utilization to other non-conventional media, such as deep eutectic solvents [39].

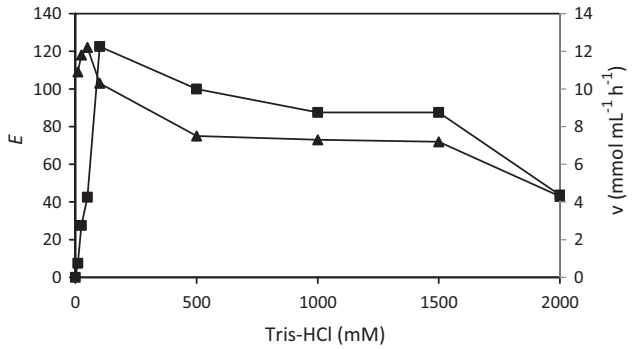
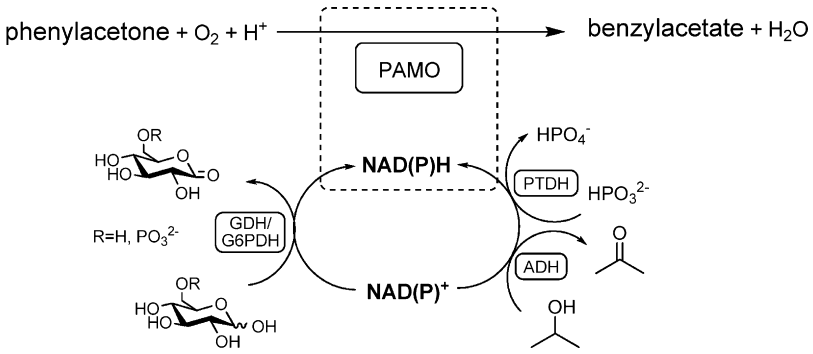


Fig. 1. Effect of the Tris/HCl pH 9.0 ionic strength in the enantioselectivity (▲) and in the space time yield (v), expressed as mmol of ketone consumed per mL and h^{-1} (■) when (±)-**1a** was oxidized by PAMO at 20 °C.



Scheme 2. Enzymatic regeneration systems for PAMO-catalyzed oxidation.

3.3. Analysis of enzymatic cofactor regeneration systems for the PAMO-catalyzed oxidations

Due to the fact that PAMO accepts some organic cosolvents for its biocatalytic activity [40,41], the effect of isopropanol (^{i}PrOH) concentration was optimized when testing the alcohol dehydrogenase TBADH in phenylacetone (**2a**) oxidation. The highest activity was found at ^{i}PrOH concentrations of 10% (v/v) (65% after 1 h). Increasing the amount of cosolvent led to a slight loss in the formation of **2b**, with an almost complete loss of activity at 50% (v/v) ^{i}PrOH.

PAMO-catalyzed oxidation of phenylacetone was subsequently studied when coupled to several enzymatic ancillary systems (glucose/GDH, glucose-6-phosphate/G6PDH, ^{i}PrOH/TBADH, sodium phosphite/PTDH or CRE2-PAMO) as shown in Scheme 2. When **2a** was oxidized at pH 8.0 and 20 or 30 °C, the highest space time yield was obtained when using GDH (13 and 40 mmol L^{-1} h^{-1}, resp.) (Fig. 2). This enzyme led to faster oxidations than G6PDH and PTDH, in either free or fused preparations. On the other hand, oxidation of **2a** catalyzed by PAMO occurred with low activity when coupled to TBADH.

The enzymatic cofactor regenerating systems were also explored at pH 9.0 and 30 °C. G6PDH led to the fastest oxidations, as indicated in Fig. 2, showing a higher activity at pH 9.0 than at pH 8.0. Oxidation of **2a** catalyzed by the PAMO/GDH system occurred with a space time yield of 20 mmol L^{-1} h^{-1}, resulting in an important loss when compared with conversions at lower pH. The two phosphite dehydrogenase systems (PTDH and CRE2) presented comparable values of space time yields at pH 8.0 and 9.0. Again, the lowest activity was obtained with TBADH.

In order to analyze the effect of the different enzymatic regeneration systems on PAMO selectivity in more detail, racemic ketones (±)-3-phenylbutan-2-one [(±)-**1a**] and (±)-4-phenyl-3-methylbutan-2-one [(±)-**3a**] and a prochiral sulfide as thioanisole (**4a**) were analyzed. PAMO-catalyzed resolution of (±)-**1a** at pH 9.0 and 30 °C coupled with GDH (Table 2) led to a 23% of (*S*)-**1b** after 20 min in a process with a good selectivity (E = 88). Baeyer–Villiger oxidation in presence of G6PDH, PTDH or CRE2-PAMO led to lower conversions, but higher selectivities, since in all cases E > 100 were obtained. The same behavior was observed in the PAMO-catalyzed kinetic resolution of (±)-**3a**. Oxidation was faster and less selective with GDH (c = 46% after 2 h and E = 29), whereas in the presence of G6PDH and the two PTDH systems (*R*)-**3b** was obtained with 50% of conversion after 3.5–4 h and $E \sim 40$.

The effect of the type of cofactor regeneration system was also observed in the sulfoxidation reactions. Thus, oxidation of thioanisole (**4a**) catalyzed by PAMO at pH 9.0 affords the highest conversion when employing glucose/GDH as NADPH-recycling system (c = 95%), while (*S*)-methyl phenyl sulfoxide [(*S*)-**4b**] presented a slightly lower optical purity than for the rest of enzymes studied (ee = 35%). In this case, the use of G6PDH and PTDH led to better conversions, close to 80%, while 60% of (*S*)-**4b** was obtained when employing the fused CRE2-PAMO. For the three enzymes, the sulfoxide was obtained with optical purities around 40%. The variation of selectivity depending on the cofactor regeneration system has been previously described [42]. Hall et al. have attributed this behavior to an allosteric effect on the enzyme.

3.4. Effect of cofactor concentration in the oxidation of ketones and sulfides catalyzed by PAMO

It is well known that the stoichiometric use of NADPH not only adversely affects the efficiency of the biocatalysts, but also increases costs of the large-scale synthetic processes. On the other hand, previous studies have revealed that $NADP^+$ binding is essential to maintain the enantioselectivity of PAMO while increasing the stability of the enzyme [43]. To probe the effect of cofactor concentration, an initial set of experiments were carried out by oxidizing phenylacetone at pH 9.0 and 30 °C when employing PAMO coupled to G6PDH at different NADPH concentrations (from 2.0 μM to 1.0 mM). As can be observed in Fig. 3, the use of lower amounts of cofactor led to an important increase in both TTN and TOF values. Thus, when phenylacetone was oxidized in the presence of 0.2 mM NADPH (the chosen concentration employed in previous studies for PAMO-catalyzed oxidations) a TTN of 44 and a TOF of 176 h^{-1} was obtained. The highest TTN (2400) and TOF (9600 h^{-1}) values were reached when phenylacetone has been oxidized in the presence of only 2.0 μM of NADPH. Thus, it is shown that PAMO-catalyzed Baeyer–Villiger oxidation can take place with the highest effectiveness at very low concentrations of cofactor.

The same study was also performed for the oxidation of racemic (±)-3-phenylbutan-2-one. As indicated in Fig. 3, the highest

Table 2
Biocatalytic properties of PAMO in the oxidation of racemic ketones (±)-**1a** and (±)-**3a** when using different NADPH enzymatic regeneration systems.[a]

Ketone	Coenzyme	t (h)	ee (%)[b] **1a–3a**	ee (%)[b] **1b–3b**	c (%)[c]	E[d]
(±)-**1a**	GDH	0.33	30	97	23	88
(±)-**1a**	G6PDH	1	45	97	32	102
(±)-**1a**	PTDH	1	42	97	30	115
(±)-**1a**	CRE2	1	21	98	18	106
(±)-**3a**	GDH	2	74	86	46	29
(±)-**3a**	G6PDH	3.5	99	78	56	42
(±)-**3a**	PTDH	4	98	80	55	40
(±)-**3a**	CRE2	4	88	87	50	43

[a] Reaction conditions: 50 mM Tris–HCl pH 9.0; 30 °C.
[b] Determined by GC.
[c] Conversion, $c = ee_s/(ee_s + ee_p)$.
[d] Enantiomeric ratio, $E = \ln\{(1-ee_s)/[1+(ee_s/ee_p)]\}/\ln\{(1+ee_s)/[1+(ee_s/ee_p)]\}$ (see Ref. [36]).

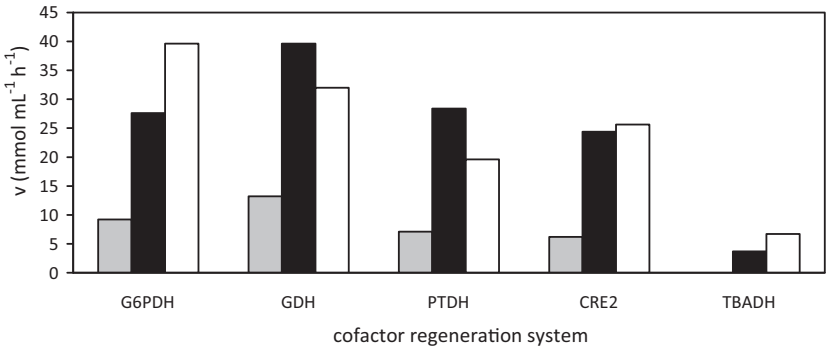


Fig. 2. Space time yields in the PAMO-catalyzed bioxidation of phenylacetone by employing different enzymatic NADPH regeneration systems when working at different reaction conditions: (i) pH 8.0 and 20 °C (grey figures); (ii) pH 8.0 and 30 °C (black figures); and (iii) pH 9.0 and 30 °C (white figures).

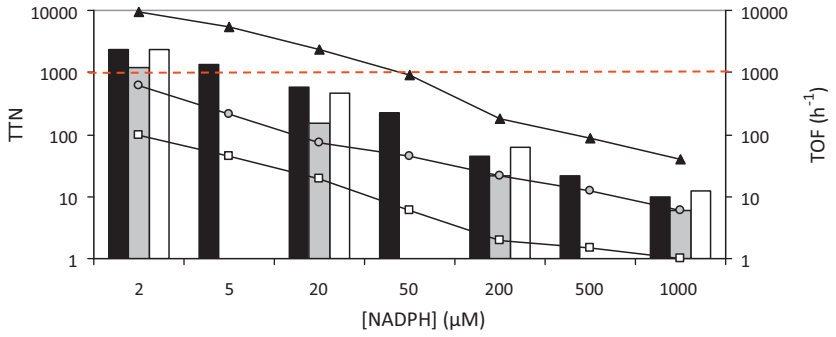


Fig. 3. Efficiency of the biocatalytic system with decreasing amounts of NADPH. TTN (bars) and TOF (lines) are represented for the oxidation of phenylacetone (black bar, △), (±)-3-phenylbutan-2-one (grey bar, ○) and thioanisole (white bar, □) catalyzed by PAMO at pH 9.0 and 30 °C.

values were achieved at 2.0 μM of NADPH (TTN = 1222 and TOF = 611). Moreover, it was observed that selectivity of the process remains virtually unaltered ($E = 100 \pm 2$), only a slight decrease was detected in PAMO selectivity at low NAPDH concentrations, close to the $K_{M,NADPH}$ (0.7 μM).

Finally, thioanisole was employed as model substrate for analyzing the effect of cofactor concentration in enzymatic sulfoxidations. As expected, lower TOF values were measured when compared with the BV oxidation of aromatic ketones, while the same trend was observed. The use of 1.0 mM or 0.2 mM NADPH led to low TOF (1 and 2 h^{-1}, respectively), while employing 2.0 μM of this cofactor increased the efficiency 100-fold, reaching a TTN value of 2405.

4. Conclusions

Different parameters that can affect the biocatalytic properties of isolated PAMO were studied for optimizing Bayer–Villiger reactions and sulfoxidations. The buffer type and ionic strength has a great influence on the activity and selectivity of PAMO. The highest *E* values combined with good conversion were achieved when working with Tris or phosphate buffers. Furthermore, PAMO was able to maintain a great percentage of its activity even at high saline concentrations (2.0 M) which underscores the applicability of this biocatalyst in non-conventional media such ionic liquids or deep eutectic solvents. The coupling of different dehydrogenases as cofactor regeneration systems clearly influences the oxidative processes catalyzed by PAMO. Depending on the reaction conditions, the best results were obtained using GDH, G6PDH or PTDH to regenerate NADPH. GDH is more active and leads to higher conversions in BVMO-catalyzed reactions, while the use of PTDH or G6PDH results in more selective oxidation process. Finally, the amount of cofactor employed in the oxidation catalyzed by PAMO in the presence of G6PDH has also been optimized. It was found that the system performed optimal at concentrations of 2.0 μM NADPH. Such a low cofactor concentration led to an increase in TTN and TOF by more than 50 times when compared with concentrations used normally. The optimal condition yielded TTN values higher than 10^3, minimum value required for making an economically reliable process.

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