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Biocatalytic properties of Baeyer–Villiger monooxygenases in aqueous–organic media

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Abstract

The biocatalytic properties of three Baeyer–Villiger monooxygenases (phenylacetone monooxygenase, 4-hydroxyacetophenone monooxygenase and ethionamide monooxygenase) in a variety of aqueous–organic media were studied using organic sulfides as substrates. The influence of the nature and the concentration of the solvents, as well as of the substrates, on the activity and enantioselectivity of the enzymes was investigated in detail. Solvents were found to decrease, to a different extent, enzyme activity. High increases of enantioselectivity and also reversal of enantiopreference were observed depending on the enzyme and on the nature of the solvent and the substrate employed.
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Keywords: Baeyer–Villiger monooxygenases; Aqueous–organic media; Organic sulfides; Chiral compounds; Enzyme stability

1. Introduction

In nature, enzymes function in aqueous solutions. However, a majority of the substrates of interests to organic chemists are likely to be hydrophobic, requiring the addition of organic solvents to achieve higher solubilities. Moreover, it has been shown that some enzymes can act as catalysts in low water-containing organic solvents to carry out processes difficult to effect in water alone [1,2]. The latter systems are especially advantageous to transform substrates that are unstable or poorly soluble in water and, in addition, at low water activity many side-reactions can be prevented, including the denaturation of enzymes which, in organic media, show higher thermal stability. Furthermore, enzyme selectivity can be markedly affected and even reversed by the solvent, providing a sort of "medium engineering" alternative to protein engineering [1]. Often, a major drawback when working with enzymes in organic solvents is the lower catalytic activity shown by the biocatalyst in these media compared to water [3]. However, in the last years, several effective techniques for enzyme activation in organic solvents have been developed [4–6].

Applications of enzymes in organic media have been studied to a great extent. Besides being used in pure organic solvents as medium, enzymes can also be applied in mixtures of water and water-miscible organic solvents [7–10] or in biphasic systems consisting of water and water-immiscible organic solvents [11–13]. In these latter systems, the enzyme and hydrophilic compounds are contained in the aqueous phase while the hydrophobic compounds are presented in the organic one. By working in media different from water, the biocatalysts are subjected to a number of factors that can alter its native, aqueous-based, structural and catalytic properties.

The use of organic solvents in enzymatic reactions has been extensively described and analyzed for several types of biocatalysts, including oxidoreductases [14,15]. However, until now, no studies using water–organic solvent systems have been performed with Baeyer–Villiger monooxygenases (BVMOs). These NAD(P)H-dependent oxidative flavoenzymes are able to incorporate one atom of molecular oxygen into the substrates, the other atom being reduced to water [16]. BVMOs catalyze a wide variety of oxidative reactions such as Baeyer–Villiger oxidations and sulfoxidations [16–19], yielding valuable compounds such as chiral lactones [20,21] and sulfoxides [22,23]. A great number of chemical methods for the synthesis of these compounds have been reported. However, a BVMOs-based approach offers the advantage of

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high enantioselectivities in environmentally benign reaction conditions.

For a long time, few BVMOs were known and only one member (cyclohexanone monooxygenase) of this class of enzymes was cloned, overexpressed and extensively studied [24–26]. This has limited the synthetic application of these biocatalysts. In recent years, a number of genes that encode BVMOs have been sequenced and the corresponding enzymes overexpressed, increasing the potentialities of this type of enzymes for synthetic purposes [19].

In the present report, we describe the use of aqueous–organic solvent media to carry out the oxidation of organic sulfides exploiting three isolated Baeyer–Villiger monooxygenases, namely: phenylacetone monooxygenase (PAMO) from *Thermobifida fusca* [27,28], 4-hydroxyacetophenone monooxygenase (HAPMO) from *Pseudomonas fluorescens* ACB [29–31] and ethionamide monooxygenase (EtaA) from *Mycobacterium tuberculosis* [32].

2. Experimental

2.1. Material and methods

PAMO, HAPMO and EtaA were obtained as previously described [27,29,32]. One unit of BVMO oxidizes 1.0 μmol of thioanisole (**1**) to methyl phenyl sulfoxide (**1a**) per minute at pH 9 and 25 °C in the presence of NADPH. Glucose-6-phosphate dehydrogenase (170 U mg^{-1}) from *Leuconostoc mesenteroides* was obtained from Fluka–BioChemika. Glucose-6-phosphate, $NADP^+$, NADPH, sulfides **1**, **2**, **3**, sulfoxides (±)-**1a**, (±)-**2a** and phenylacetone **7** were purchased from Sigma–Aldrich–Fluka. Benzyl sulfides **5** and **6** were products from Lancaster. Phenyl propyl sulfide was prepared according to ref. [33]. Sulfoxides (±)-**3a**–**6a** were prepared by chemical oxidation of the corresponding sulfides and exhibited physical and spectral properties in accord with those reported [33,34]. All other reagents and solvents were of the highest quality grade available and were from Aldrich–Fluka.

Chemical reactions were monitored by analytical TLC, performed on Merck silica gel 60 F_{254} plates and visualized by UV irradiation. Flash chromatography was carried out with silica gel 60 (70–230 mesh, Merck). Chiral HPLC analyses were conducted on a Jasco HPLC instrument (model 880-PU pump, model 870-UV/VIS detector) equipped with a Chiralcel OD (Daicel) or a Chiralcel OB (Daicel) chiral column. Acetanilide was used as internal standard to determine the conversion of the oxidation processes. The kinetic measurements were carried out with a Jasco V-530 UV spectrophotometer.

Absolute configurations of the chiral sulfoxides were established by comparison of the HPLC chromatogram profile with the patterns described in previous experiments for the compounds with known configurations.

2.2. General method for the enzymatic oxidation of sulfides *1*–*6*

The sulfides **1**–**6** (0.02 mmol, 1.0 equiv.) were dissolved in 50 mM Tris/HCl pH 9.0/organic cosolvent system (1.0 mL), containing glucose-6-phosphate (0.04 mmol, 2.0 equiv.), glucose-6-phosphate dehydrogenase (10.0 units), NADPH (0.2 mM), acetanilide (0.02 mg) and a Baeyer–Villiger monooxygenase (1.0 unit). The mixture was shaken at 250 rpm and 25 °C in a rotatory shaker for the times indicated in Table 2. The reaction was then stopped, worked up by extraction with dichloromethane (3 × 0.5 mL), dried over Na_2SO_4 and analyzed by chiral HPLC in order to determine the conversion and the enantiomeric excess of sulfoxides **1a**–**6a**. For all the solvents tested, control experiments in the absence of enzyme resulted in no conversion.

2.3. PAMO activity and stability measurements

The enzymatic activity was assayed in 50 mM Tris/HCl pH 9.0/organic cosolvent mixtures (1.0 mL) containing 1.0 mM phenylacetone, 0.1 mM NADPH and 0.1 unit of PAMO, by monitoring NADPH consumption at 340 nm ($\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$). The experiments were carried out at 25 °C using air-saturated buffers.

The enzyme stability measurements were performed by incubating at 25 °C and 250 rpm in a rotatory shaker, a 1.0 unit solution of PAMO in 50 mM Tris/HCl pH 9.0/cosolvent media (9.8 mL). At different times, samples were taken and after addition of phenylacetone (1.0 mM) and NADPH (0.1 mM), the activity was measured by monitoring NADPH consumption at 340 nm.

2.4. Glucose-6-phosphate dehydrogenase activity measurements

The enzymatic activity was assayed in 50 mM Tris/HCl pH 9.0/methanol mixtures (1.0 mL), containing 1.0 mM glucose-6-phosphate, 0.1 mM $NADP^+$ and 0.1 unit of glucose-6-phosphate dehydrogenase, by monitoring NADPH formation at 340 nm. The experiments were carried out at 25 °C using air-saturated buffers.

3. Results and discussion

3.1. PAMO catalyzed oxidations

The effects of a broad range of organic solvents, with different physico-chemical characteristics, on the catalytic properties of PAMO were investigated.

3.1.1. PAMO oxidation of thioanisole (*1*) in the presence of 30% organic cosolvent

A first set of experiments was carried out using thioanisole (**1**) as substrate and a 50 mM Tris/HCl buffer pH 9, containing 30% of organic cosolvent as the reaction medium (Table 1). The NADPH cofactor consumed in the PAMO catalyzed oxidations was regenerated by the ancillary enzymatic system glucose-6-phosphate/glucose-6-phosphate dehydrogenase (Scheme 1).

When PAMO catalyzed oxidation of **1** was carried out in buffer alone, (*R*)-**1a** was obtained with 94% of conversion and a moderate optical purity (ee = 43%, Table 1).

Table 1
Effect of organic cosolvent (30%, v/v) on BVMOs catalyzed oxidation of thioanisole (**1**)

Entry	Cosolvent	PAMO		HAPMO		EtaA	
		c (%)[a]	ee (%)[a]	*c* (%)[a]	ee (%)[a]	*c* (%)[a]	ee (%)[a]
1	None	94	43 (*R*)	96	≥98 (*S*)	43[b]	33 (*S*)
2	MeOH	32	89 (*R*)	≤3	–	10	84 (*S*)
3	EtOH	9	48 (*R*)	≤3	–	4	74 (*S*)
4	*n*-PrOH	5	39 (*R*)	≤3	–	≤3	–
5	*i*-PrOH	13	39 (*R*)	8	94 (*S*)	≤3	–
6	BuOH	≤3	–	≤3	–	≤3	–
7	2-OctOH	≤3	–	32	93 (*S*)	≤3	–
8	Glycerol	35	32 (*R*)	23	≥98 (*S*)	6	50 (*S*)
9	Acetone	8	12 (*R*)	≤3	–	≤3	–
10	1,4-dioxane	37	8 (*R*)	≤3	–	5	8 (*S*)
11	CH_3CN	8	31 (*R*)	5	32 (*S*)	≤3	–
12	$^iPr_2O$	7	42 (*R*)	61	≥98 (*S*)	≤3	–
13	^{t}BuOMe	10	12 (*R*)	41	≥98 (*S*)	11[c]	26 (*R*)
14	$CHCl_3$	≤3	–	≤3	–	≤3	–
15	AcOEt	≤3	–	≤3	–	<3	–
16	Toluene	≤3	–	23	74 (*S*)	≤3	–
17	Hexane	23	43 (*R*)	35	97 (*S*)	≤3	–
18	Thioanisole	n.d.	38 (*R*)	n.d.	≥98 (*S*)	n.d.	26 (*S*)

n.d.: not determined; for reaction conditions, see Section 2.
[a] Determined by HPLC.
[b] Reaction stopped after 16 h.
[c] Reaction carried on with 3 units of EtaA.

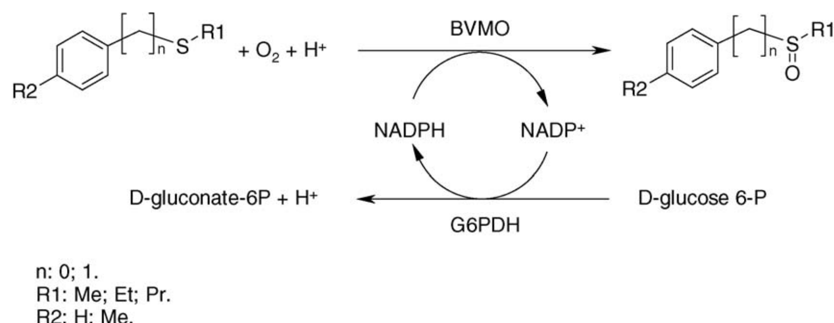
Surprisingly, when the reaction was conducted with 30% methanol (entry 2), (*R*)-**1a** was obtained with a moderate conversion (32% after 24 h) but with a relatively high enantioselectivity (ee = 89%). Also with ethanol (entry 3) the selectivity was slightly higher than in buffer, but for this solvent the conversion was even lower. With propanol, isopropanol and glycerol, no selectivity improvement was found. Practically no reaction was observed with the water-immiscible alcohols 1-butanol and 2-octanol.

Oxidation of thioanisole was carried out with other polar or apolar solvents (entries 9–17). With 30% acetone, both the optical purity and the conversion were very low. With the other solvents tested (Table 1), low conversions or selectivities or both were obtained.

The biocatalytic process was also conducted using 30% of substrate **1** (entry 18). The enantiomeric excess of (*R*)-**1a** was very similar to that in buffer, which indicates that high concentrations of substrate do not affect enzyme selectivity.

*3.1.2. Parameters affecting PAMO biooxidation of **1** in methanol*

Once it was established that methanol was the best cosolvent from the point of view of enantioselectivity, the role of this solvent was studied in more detail. First, the effect of methanol concentration tested in the 0–50% range was analyzed (Fig. 1). An almost linear decrease of reaction rate as a function of methanol was observed, with 8% of conversion when the reaction medium contained 50% cosolvent. The effect on PAMO enantioselectivity was more interesting. Addition of only 1% methanol produced a remarkable improvement in (*R*)-methyl phenyl sulfoxide optical purity (ee = 68%), indicating that even a small amount of cosolvent can induce an important change in the biocatalyst properties. Increase in solvent concentration up to a 10% increased the enantioselectivity up to ee = 89% for (*R*)-**1a**. Further increases of methanol concentration up to 50% did not induce any improvement in selectivity.



Scheme 1. BVMO oxidation of organic sulfides in aqueous–organic media.

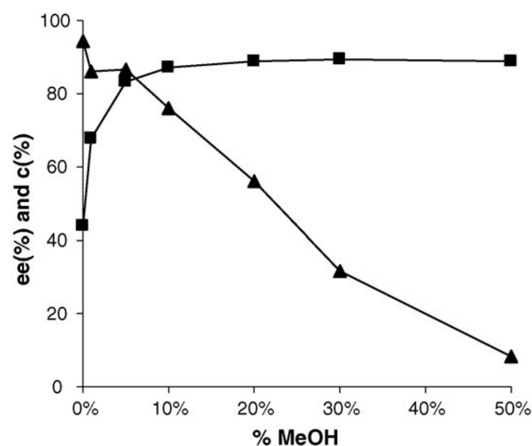


Fig. 1. Effect of methanol concentration on the degree of conversion (*c*) (▲) and on the ee of (*R*)-**1a** (■) in PAMO catalyzed oxidation of thioanisole (**1**). Reaction time: 24 h.

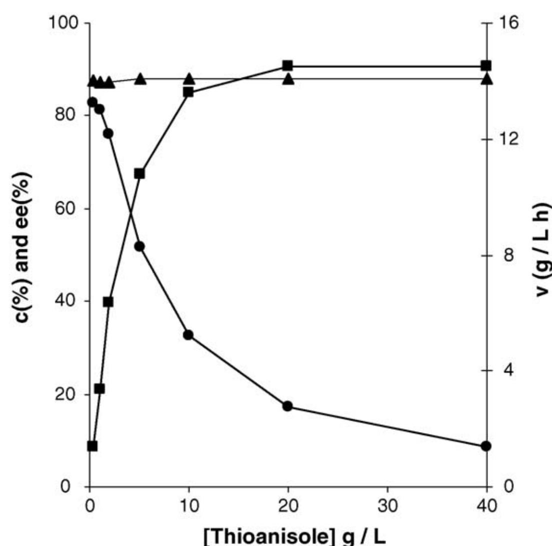


Fig. 2. Effect of thioanisole concentration on the degree of conversion (●), on the ee of the product (▲) and on the reaction rate (■) in PAMO oxidation of **1** in presence of 10% methanol. The reaction rates (*v*) were expressed as grams of substrate transformed per liter per hour. Reaction time: 24 h.

The influence of thioanisole concentration on the biocatalytic reaction was studied in presence of 10% methanol and monitored at 24 h (Fig. 2). At all the concentrations tested ($0.4–40\,g\,L^{-1}$), the enantiomeric excess of (*R*)-methyl phenyl sulfoxide remained constant (ee = 88%). As expected, when the concentrations were higher the conversions were lower. The reaction rates increased as a function of thioanisole with a hyperbolic behavior, and the maximum was reached at a substrate concentration of about $20\,g\,L^{-1}$.

3.1.3. PAMO catalyzed oxidation of other organic sulfides in 30% MeOH

Once it was found that the highest selectivity in the oxidation of thioanisole was achieved in methanol, the study was extended to other organic sulfides that had shown a moderate or low selectivity in aqueous media (Table 2) [35]. As expected, the conversions in 30% methanol were much lower than those observed in buffer alone for all the substrates tested. Instead, the ee of (*R*)-methyl *p*-tolyl sulfoxide (*R*)-**2a** increased from 11 to 81% when replacing pure aqueous buffer with buffer containing 30% methanol, as shown in entries 3–4. Surprisingly, in the oxidation of sulfides **3** and **4**, the remarkable improvement in the optical purities of sulfoxides (*R*)-**3a** and **4a** in the presence of methanol was coupled with a change in enzyme enantiopreference, which changed from (*S*) in buffer to (*R*) in 30% methanol (entries 5–8). In view of these results, the enzymatic oxidation in 30% methanol was also performed with benzyl sulfides **5** and **6**, for which it was possible to obtain high enantiomeric excesses (ee > 94%) also in absence of cosolvent. For these two compounds, however, no change in either enantioselectivity or enantiopreference was observed.

3.1.4. PAMO activity and stability in aqueous–organic media

The effect of organic cosolvents (at 10 or 30% concentration) on the initial activity of PAMO was studied using phenylacetone, the best substrate described for PAMO so far [27].

The rate of phenylacetone oxidation to benzyl acetate by PAMO at pH 9 was $3.4 \pm 0.3\,s^{-1}$ (Table 3). The presence of organic cosolvents caused a significant loss in initial activity. Nevertheless, substantial initial rates were observed with all solvents tested. Methanol was the solvent with only a limited effect on the enzyme activity (75 and 60% of remaining activity in 10 and 30% methanol, entries 2 and 3). Also ethanol and 1,4-dioxane had limited effects on enzyme activity (entries 4–7). Instead, with propanol and acetonitrile only 10–20% of activity was left (entries 11 and 13). The enzyme showed good activity in isopropanol and acetone (entries 8, 9 and 12). Concerning this latter solvent, it should be mentioned that acetone is a PAMO substrate. In fact, when the reaction was carried out in 10% acetone, but in absence of phenylacetone, an apparent activity of $0.2\,s^{-1}$ was observed (entry 10).

The effect of methanol on the activity of the glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, the enzyme used as NADPH regeneration system, was also studied. Only a slight decrease in activity was observed when working at 10 or 30% methanol/aqueous buffer solutions (respectively, 89 and 82% of activity in buffer).

The stability of PAMO in presence of organic cosolvents was also studied (Fig. 3). In buffer only, the enzyme was stable for more than 70 h at 25 °C. In the presence of 10% methanol or 1,4-dioxane about 50% of the original activity was retained after 48 h. Instead, 30% of cosolvent induced a rapid destabilization of PAMO with all tested solvents, especially in the case of ethanol.

3.2. HAPMO catalyzed oxidations

3.2.1. Oxidations of thioanisole in aqueous–organic media

As described in previous work, HAPMO efficiently catalyzed oxidation of thioanisole in Tris/HCl buffer yielding enantiopure (*S*)-**1a** with almost complete conversion [30]. The effects of solvents (30%, v/v) on this process are shown in Table 1. As in the case of PAMO, HAPMO activity was in general quite sen-

Table 2
PAMO catalyzed oxidation of organic sulfides in presence of 30% methanol

Entry	Sulfide structure	MeOH (%)	*t* (h)	*c* (%)[a]	ee (%)[a]	Configuration
1	$C_6H_5SCH_3$ (**1**)	None	24	94	44	*R*
2		30	24	32	89	*R*
3	*p*-$CH_3C_6H_5SCH_3$ (**2**)	None	24	68	11	*R*
4		30	24	10	81	*R*
5	$C_6H_5SCH_2CH_3$ (**3**)	None	24	79	33	*S*
6		30	24	26	87	*R*
7	$C_6H_5SCH_2CH_2CH_3$ (**4**)	None	24	56	19	*S*
8		30	24	9	56	*R*
9	$C_6H_5CH_2SCH_3$ (**5**)	None	6	29	94	*S*
10		30	18	18	94	*S*
11	$C_6H_5CH_2SCH_2CH_3$ (**6**)	None	6	36	98	*S*
12		30	18	20	≥99	*S*

For oxidation conditions, see Section 2.
[a] Determined by HPLC.

Table 3
Effect of organic cosolvents on PAMO activity with phenylacetone **7** as substrate

Entry	Cosolvent	% Cosolvent	k_{obs} (s^{-1})[a]	Relative activity (%)
1	None	–	3.4	100.0
2	MeOH	10	2.6	75.1
3	MeOH	30	2.0	59.6
4	EtOH	10	1.9	55.1
5	EtOH	30	1.6	47.9
6	1,4-Dioxane	10	2.3	66.8
7	1,4-Dioxane	30	1.6	47.0
8	Acetone	10	1.9	55.2
9	Acetone	30	1.3	38.1
10	Acetone[b]	10	0.2	7.0
11	PrOH	30	0.7	19.6
12	*i*-PrOH	30	1.3	48.4
13	Acetonitrile	30	0.4	11.5

[a] Standard error lower than 10%.
[b] Oxidation carried on in absence of phenylacetone.

sitive to the solvent. However, the solvents that mostly affected the activity did not always coincide for the two enzymes. For HAPMO, no reaction was observed with the alcohols tested, with the exception of ^{i}PrOH, 2-octanol and glycerol. Also other polar

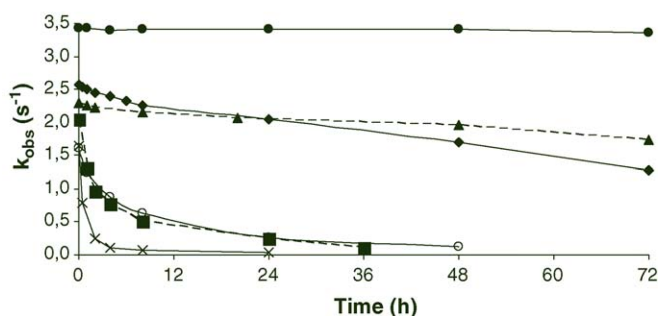


Fig. 3. PAMO stability in aqueous–organic media at 25 °C, 50 mM Tris/HCl pH 9 (●) or in buffer containing 10% (♦) or 30% methanol (■), 10% (▲) or 30% (○) 1,4-dioxane, and 30% ethanol (×). The activity was measured at different times using phenylacetone as substrate.

water-miscible solvents gave no reaction (entries 9–11, Table 1). With ethers such as ^{i}Pr$_2$O or ^{t}BuOMe optically pure (*S*)-methyl phenyl sulfoxide was obtained with high degrees of conversion, especially in the case of ^{i}Pr$_2$O (61%, entry 12, Table 1). As for PAMO, chloroform and ethyl acetate completely inactivated the enzyme. Thioanisole at 30% concentration did not affect HAPMO selectivity, resulting in formation of enantiopure *S* sulfoxide.

3.2.2. Effect of substrate concentration in HAPMO catalyzed oxidation of 1 in presence of 30% ^{i}Pr$_2$O

As reported in the previous paragraph, the best cosolvent in HAPMO oxidation of **1** was found to be ^{i}Pr$_2$O. In this case, differently from PAMO with methanol, the system is biphasic. As a result, the concentration of the substrates and products in the aqueous phase, where the biocatalysis takes place, depends not only on their global concentration but also on their partition coefficient. For this reason, the oxidation process was studied using different thioanisole concentrations. As shown in Fig. 4, the enantiomeric excess of the product remained high (ee ≥ 98%) and constant for the entire range of concentrations tested. By increasing the concentration of **1**, the reaction rate increased reaching a maximum at a concentration of 14 g L^{-1}. Higher concentrations of thioanisole produced a negative effect, decreasing the maximum reaction rate. It should be emphasized that this phenomenon was not observed with PAMO (Fig. 2).

3.3. EtaA catalyzed oxidations

The oxidation process in aqueous–organic media was also tested with EtaA. This biocatalyst was able to oxidize sulfide **1** to (*S*)-**1a** with moderate selectivity (ee = 33%) and reactivity (*c* = 43% after 16 h) (Table 1) [36]. As shown for PAMO, the addition of methanol resulted in an important increase in enzyme selectivity, yielding (*S*)-methyl phenyl sulfoxide with relatively high optical purity (ee = 84%). Ethanol and glycerol also improved the enzyme enantioselectivity, but to a lesser extent (ee of 74 and 50%, respectively). In all three cases, the

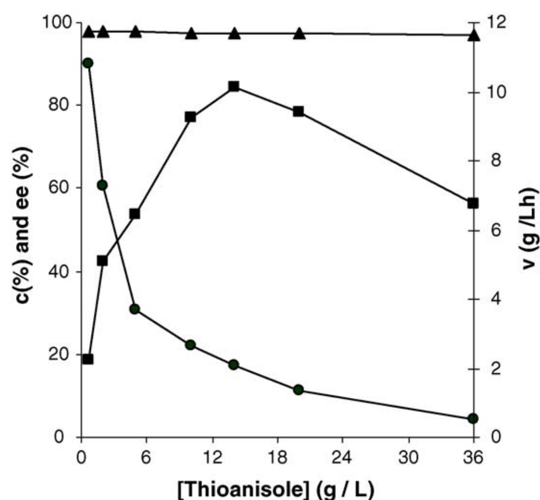


Fig. 4. Effect of thioanisole concentration on the degree of conversion (●), on the ee of the product (▲), and on the reaction rates (■), in the HAPMO catalyzed oxidation of **1** in presence of 30% $^{i}Pr_2O$. Reaction rates (v) were expressed as grams of substrate transformed per liter per hour. Reaction time: 24 h.

solvents inhibited the conversion, especially for these two latter alcohols. With the other solvents tested with EtaA, very low or no reaction was observed. The most interesting result was achieved with ^{t}BuOMe (entry 13, Table 1) where mainly the (*R*)-sulfoxide instead of the (*S*)-sulfoxide was obtained. So, by changing the solvent used in EtaA-catalyzed oxidations, we were able to improve the EtaA enantioselectivity (using MeOH), or revert EtaA enantiopreference (using ^{t}BuOMe) towards thioanisole.

4. Conclusions

It was demonstrated for the first time that oxidation processes catalyzed by BVMOs can be carried out in a variety of aqueous–organic media. The organic solvents were found to generally decrease, at varying extent, enzyme activity. This negative effect was partly counterbalanced by the increase of solubility of sulfide substrates in these media compared to the one in aqueous buffer. Interestingly, significant improvements of enantioselectivity in thioanisole oxidation by PAMO and EtaA were induced by short chain alcohols such as methanol and ethanol. Remarkably, methanol was able to cause a reversal of PAMO enantiopreference in the case of a few substrates. Reversal of enantiopreference was also observed with EtaA when using ^{t}BuOMe. Such an effect of organic solvent on the enantioselectivity of a specific enzyme has been reported for a number of biocatalysts [1,37], especially hydrolases. In some of these cases, a correlation between the properties of the solvents as hydrophobicity, dipole moment or dielectric constant and the enantioselectivity was demonstrated [38]. However, for the BVMOS described in this study, no evident correlation between the physico-chemical properties of the solvents and the effect on enantioselectivity was found. It might be hypothesized that in these enzymes solvents exert their influence on enantioselectivity by the following mechanism [38]: solvents molecules bind in or near the enzyme active site and depending on their structure, interfere with the association of the substrate. In the case of BVMOs, this will influence the positioning of the substrate with respect to the reactive hydroperoxyflavin that oxidizes the sulfur moiety. This agrees with the observation that solvents can bind specifically in enzyme active sites, e.g. in the case of subtilisin and chymotrypsin [39].

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