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Comparison of cyclohexanone monooxygenase as an isolated enzyme and whole cell biocatalyst for the enantioselective oxidation of 1,3-dithiane

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Abstract

Both whole cells of recombinant *Escherichia coli* TOP10, overexpressing cyclohexanone monooxygenase (CHMO) and isolated cyclohexanone monooxygenase, were used to carry out the enantioselective oxidation of 1,3-dithiane (**1**) to (*R*)-1,3-dithiane-1-oxide (**2**). The two biocatalysts were evaluated under various experimental conditions (e.g., shaken flask or bioreactor; non-bound or resin-adsorbed substrate; different substrate concentrations) in terms of volumetric productivity and enantioselectivity. While productivity was similar in the two cases (up to $0.58\,g\,L^{-1}\,h^{-1}$), the optical purity of the product was much higher with the isolated enzyme (up to 98% e.e.) than with the whole cell biocatalyst (up to 85% e.e.).
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1. Introduction

In recent years biocatalytic processes, using whole cells or isolated enzyme bicatalysts, have found increasingly widespread application [1,2]. This is particularly true in the pharmaceutical and agrochemical industries, where the need for optically pure molecules is critical [3]. Many of the most useful conversions concern the synthesis of products with one or more chiral centres, where the use of biocatalysts circumvent the need for multi-step procedures in order to achieve enantiomerically pure products. Of particular interest are oxidative biotransformations, due to the high regio- and stereo-selectivity that can often be achieved in the synthesis of industrially useful synthons [4–6].

An excellent example of a useful oxidative product class is the chiral sulfoxides, which have experienced an enormous expansion in the past decade from both a quantitative and qualitative perspective [7]. Chiral sulfoxides are important compounds because they are both valuable synthons [8] and versatile chiral auxiliaries [9] in synthetic chemistry. Furthermore, among natural products, they occur in functionalised amino acids that exhibit a wide range of biological activities including antibiotic properties [10]. In addition, they are also found as metabolites of many sulfur-containing drugs [11].

Several groups of enzymes, such as monooxygenases, dioxygenases and chloroperoxidases [12], are able to asymmetrically oxidise sulfur. Among these, one of the most studied has been cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus* sp. NCIMB 9871 [12–14]. It oxidises, often with good stereoselectivities, a very wide range of dialkyl sulfides [15], benzyl alkyl sulfides [16,17], alkyl

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aryl sulfides [13,18], and dithiocetales [13,19–21]. These reactions have been carried out using either whole cell or isolated enzyme biocatalysts. Each of the two catalyst forms has advantages and disadvantages. For example, with reactions catalysed by whole cell bicatalysts there is no need for an ancillary enzymatic system for the regeneration of the co-factor (NADPH) and for enzyme isolation. On the other hand, isolated enzyme bicatalysts do not suffer from substrate mass transfer limitations and possible side reactions or overmetabolism of the product. Downstream processing might also be more straightforward and potentially substrate concentrations higher.

In the work reported here, both whole cells of *E. coli* TPO10 [pQR239], in which CHMO had been overexpressed [22], and the enzyme isolated from the cells, were used to carry out the enantioselective oxidation of 1,3-dithiane (**1**) to (*R*)-1,3-dithiane-1-oxide (**2**) (Scheme 1).

The enantioselective oxidation of **1** to **2** has already been described for catalysis by both the isolated enzyme [23] and whole cells [19–21] and the e.e. values of product **2** have been found to be in the 84–98% range. However, in all cases the concentration of the substrate in the medium was low (1–2 $g\,L^{-1}$) and, therefore, discouraging for preparative applications. It should be emphasised that, with chemical methods, enantiomerically pure (*R*)-1,3-dithiane-1-oxide can be obtained only with a three-step procedure [24], while the one-step oxidation of **1** yields **2** with only 20% e.e. [25].

The aim of the present research was to substantially improve the concentration of the substrate and product in the medium, to make the enzymatic process more synthetically useful. Furthermore, a comparison between the performance of whole cells and that of the isolated enzyme was conducted.

2. Experimental

2.1. Materials

Growth medium constituents were obtained from Oxoid (Oxoid S.p.A., Garbagnate Milanese, Milano, Italy). The reagents used in the CHMO enzyme assay and reactions were bought from Sigma-Aldrich (Poole, Dorset, UK). All other chemicals were analytical grade. The resins used in these studies were kindly donated by Resindion (Resindion S.r.l., Milano, Italy). All of the resins were commercially available.

2.2. Growth of recombinant E. coli TOP10 [pQR239]

An *E. coli* TOP10 strain, that overexpresses *Acinetobacter* sp. cyclohexanone monooxygenase under the control of the L(−)arabinose-inducible *araBAD* promoter, was used throughout these studies [26]. The plasmid also contains an ampicillin resistance marker gene. The transformed microorganism was cultivated essentially as described previously [22]. The medium (pH 7.0) contained 10 $g\,L^{-1}$ of NaCl and soybean peptone, 12 $g\,L^{-1}$ of glycerol, 25 $g\,L^{-1}$ of yeast extract and 100 $mg\,L^{-1}$ of ampicillin. Routinely, a 100 ml pre-culture was used to inoculate a 2 L fermenter (working volume 1 L) (Labfors, Infors Italia S.r.l., Italy) at 37 °C. The fermenter was stirred at 950 rpm and aerated with an airflow of 1.0 vvm via a submerged sparger. When the culture reached an OD_{600} of 8.0, L(−)arabinose (2 $g\,L^{-1}$) was added to induce the CHMO production. After 2 h growth (OD_{600} of approximately 13), the cells were harvested at 4 °C. The cell yield from a 1 L working volume fermenter was typically around 7 $g_{dcw}\,L^{-1}$.

2.3. Preparation of cyclohexanone monooxygenase

CHMO was partially purified from the *E. coli* TOP10 strain. Unless otherwise indicated, all steps of purification were carried out at 4 °C. The cells were disrupted by sonication, and cell debris removed by centrifugation. The supernatant was subjected to fractionation with $(NH_4)_2SO_4$ and the fraction which precipitated between 40 and 85% saturation was retained. This was redissolved in 0.02 M potassium phosphate buffer, pH 7.0, dialysed overnight against the same buffer and applied to an anion exchange column (Fractogel EMD DEAE, 10 cm × 2 cm) which was previously equilibrated with 0.02 M potassium phosphate buffer, pH 7.0. The enzyme was eluted with a linear gradient of 0.15 M NaCl in the same buffer, at a flow rate of 2 ml/min. Active fractions were collected, dialysed overnight against 0.02 M potassium phosphate buffer, pH 7.0 and lyophilised. The enzymatic activity was assayed by monitoring NADPH consumption at 340 nm using as the assay buffer 0.05 M Tris–HCl, pH 8.6, containing 0.6 mM methyl phenyl sulphide (thioanisole) and 0.12 mM NADPH. The enzyme had a specific activity of 2 U/mg of protein.

2.4. Adsorption of substrate and product on resins

Three resins with a decreasing degree of hydrophobicity, i.e., Sepabeads SP207, HP2MGL and HP20L were employed. To estimate the adsorption capacity of each of these resins, aqueous solutions at different concentrations of **1** and **2** were added to the resin. After few hours of gentle longitudinal shaking, the mixture was centrifuged and the supernatant was removed from the resin. The amount of resin-bound **1** or **2** was obtained, directly, by determination of the ethyl ether extract of the resin and, indirectly, by measuring **1** and **2** remained in the supernatant. The data obtained by the two methods were in good agreement. For substrate loading on the resin to be used for biotransformations, 100 ml of wet SP207 was gently shaken for 12 h in the presence of 3.2 g of 1,3-dithiane (**1**) and 900 ml of 0.05 M phosphate buffer, pH 7.0. The resin was then recovered by filtration and used in the biotransformation. Under these conditions, about 94% of **1** was bound by SP207.

2.5. CHMO kinetics

CHMO kinetic studies were conducted in 0.05 M Tris–HCl buffer, pH 8.6, 25 °C. The reaction mixture contained, besides CHMO, 0.1 mM NADPH, 5–17 mM 1,3-dithiane (for K_m determination and substrate inhibition studies). The consumption of NADPH was spectrophotometrically monitored at 340 nm.

2.6. Whole cell biotransformations

Preliminary experiments with whole cells were carried out in 250 ml Erlenmeyer flasks covered by cellulose cap and aluminium foil (working volume 25 ml), in an orbital shaker (250 rpm) at 30 or 37 °C, using as the substrate 1,3-dithiane at 1–10 g L^{-1} concentrations; cell concentrations were: 1–20 g L^{-1}. The reaction progress was monitored by HPLC as specified below. Phosphate buffer 0.05 M, pH 7.0, or directly the fermentation broth were used as the reaction media. In both cases, before starting the biotransformation, glycerol (as carbon source) was added to the reaction mixture at 5 g L^{-1} concentration.

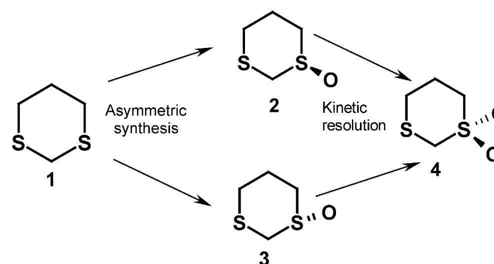
Biotransformations using whole cells (1–12 g_{dcw} L^{-1}) were also carried out at 30 °C directly in the culture medium, after growth period. Cell growth and biotransformation were time-separated to optimise cultivation and production independently. The biotransformations experiments were performed in the fermenter. Glycerol was added at 5 g L^{-1} concentration before the addition of the substrate (1–10 g L^{-1}, free or adsorbed onto the resin). The desired cell concentration was obtained either by diluting the culture with freshly sterilised culture medium or by centrifuging and resuspending the cells in the proper volume of medium. The aeration rates were 1.5 or 3.0 vvm via a submerged sparger, and the stirring rates were 400 or 700 rpm (Rushton impeller). The final reaction volume was 1 L. When the dissolved oxygen concentration in the bioreactor increased above 80% DOT, indicating carbon source depletion, 5 g L^{-1} glycerol was added. The reaction progress was monitored as specified below.

2.7. Biotransformations with isolated CHMO

1,3-Dithiane (2–20 g L^{-1}) was biooxidised, at 25 °C and under gentle stirring, in 0.05 M Tris–HCl buffer pH 8.6, containing 0.5 mM of NADP, 5% (v/v) propan-2-ol, partially purified CHMO (1500–5000 U L^{-1}) and alcohol dehydrogenase from *Thermoanerobium brockii* (ADHTB) (2000–10,000 U L^{-1}). Before use, the substrate was ground and sonicated. The reaction progress was monitored as specified below.

2.8. HPLC analysis

The biotransformation process was monitored on samples withdrawn at different times from the reaction medium and extracted three times (1/1, v/v) with ethyl ether. In the case of whole cells, the samples were freed from solids by centrifugation before extraction. The degree of oxidation of 1,3-dithiane (Scheme 1) and the enantiomeric excess of the product 1,3-dithiane-1-oxide were determined on the extracts, evaporated and redissolved in propan-2-ol, by chiral HPLC on a Chiralcel OD column (Daicel, Illkirch, France), using *n*-hexane/propan-2-ol (75:25) as the mobile phase and reading at 215 nm [23].



Scheme 1. Pathway of the CHMO-catalysed oxidation of 1,3-dithiane **1** to monosulfoxides **2** and **3** and monosulfone **4**.

3. Results and discussion

3.1. CHMO kinetics

The plot of enzyme activity as a function of 1,3-dithiane concentration showed that CHMO suffers from substrate inhibition (Fig. 1). The highest rate was obtained at about 1 mM **1** and then decreased at higher concentrations, such that at a substrate concentration of 17 mM (2 g L^{-1}) the rate was about 40% of the maximum. It was not possible to check the effect of higher substrate concentrations since the solubility of **1** in aqueous buffer was around 20 mM. The K_m of CHMO on **1** was found to be quite low at 45 ± 6 μM, while a rough estimate of the K_i gave a value of around 15 mM.

Product **2** was also found to slightly inhibit enzyme activity. For example using 1 mM substrate and 50 mM sulfoxide, the inhibition of the enzyme activity was about 20%.

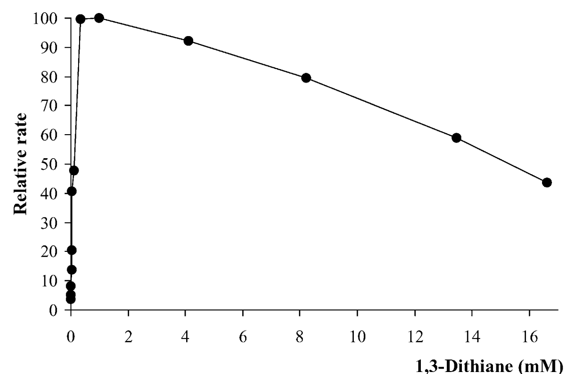


Fig. 1. Activity of CHMO as a function of substrate (**1**) concentration. For conditions, see Section 2.

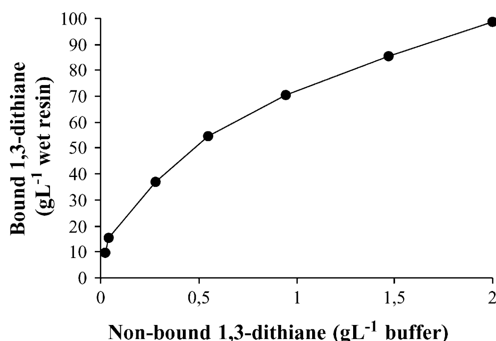


Fig. 2. Binding of 1,3-dithiane on the SP207 resin as a function of non-bound 1,3-dithiane. For conditions, see Section 2. It should be noted that 1 L of wet resin corresponds to 340 g of dry resin.

3.2. Adsorption of substrate and product on resins

Several research groups have already shown that resins, able to release the substrate in the reaction medium and/or to adsorb the formed product, can be satisfactorily used to overcome problems of low solubility or of inhibition by substrate and/or product [27–30]. Since both 1,3-dithiane and its oxidation product **2** inhibit CHMO, and considering that the solubility of **1** in aqueous buffers is rather low (about 2 g L^{-1}), this approach has been adopted also for the CHMO-catalysed oxidation of **1**.

Three resins, with different degrees of hydrophobicity, were tested as adsorbents for substrate **1** and product **2**. SP207 was the most satisfactory, with respect to adsorption rate (five-fold faster than HP2MGL and HP20L) and adsorption capacity (3- and 1.5-fold higher than HP2MGL and HP20L, respectively). The reason for this behaviour probably lies in the higher hydrophobicity of SP207. The binding, at equilibrium, of 1,3-dithiane and 1,3-dithiane-1-oxide to SP207 is shown in Figs. 2 and 3. It can be seen that the correlations between either the non-bound substrate (Fig. 2) or non-bound product (Fig. 3) concentration in the buffer and the concentration of the two compounds on the wet resin were not linear. In the range of concentrations investigated, a plateau for the binding of **1** and **2** on the resin was not reached. It can also be seen that the affinity of the substrate for the resin was higher than that of the product. For example, at 2 g L^{-1} (maximum solubility in buffer) of non-bound 1,3-dithiane, its concentration on SP207 (bound 1,3-dithiane) was of 100 g L^{-1} of wet resin (Fig. 2), whereas at the same value of non-bound 1,3-dithiane-1-oxide, its concentration on SP207 was 35 g L^{-1} of wet resin (Fig. 3).

For bioconversions, the starting conditions were such to provide 1,3-dithiane non-bound at 0.2 g L^{-1} (1.6 mM) buffer and bound at 30 g L^{-1} wet resin. These conditions were chosen in order (i) to work at the optimum substrate concentration for activity (see Fig. 1) and (ii) not to saturate the resin and, thereby, facilitate product adsorption.

3.3. Whole cell biotransformations

Whole cell biotransformations were carried out in either shaken flasks or a stirred bioreactor with substrate supplied non-bound or resin-adsorbed. When using shaken flasks and 1 g L^{-1} of non-bound 1,3-dithiane (i.e., under the conditions already described by some of us [19] and others [20]), the e.e. values of the product (*R*)-1,3-dithiane-1-oxide were up to 99%, depending on the degree of conversion [19,23]. The graph of the rate of substrate conversion versus whole cell concentration gave a bell-shaped curve (Fig. 4). These results can be explained considering that in shaken flasks aeration is limited and, therefore, at high cell concentration ($\geq$10 g L^{-1}) the greater part of the oxygen is consumed for non-productive endogenous cell respiration [6].

Higher concentrations of substrate gave less satisfactory results both in terms of conversion and optical purity. Thus, with 5 g L^{-1} of non-bound 1,3-dithiane and 5 g_{dcw} L^{-1} the conversion and product e.e. values were 57 and 82%, respectively, after 24 h reaction in a shaken flask. Better results (i.e., 84% conversion (79% formation of product **2**) and 85% e.e.) were obtained in the same conditions but using the resin-adsorbed substrate. In this case, the volumetric productivity was 0.18 g L^{-1} h^{-1}. A higher cell concentration (10 g_{dcw} L^{-1}) gave a lower conversion due to higher endogenous respiration.

To overcome problems of oxygen limitation, a stirred bioreactor was used at various aeration and agitation rates. In this case too, both non-bound and resin-adsorbed substrates were used, as well as various cell concentrations. Table 1

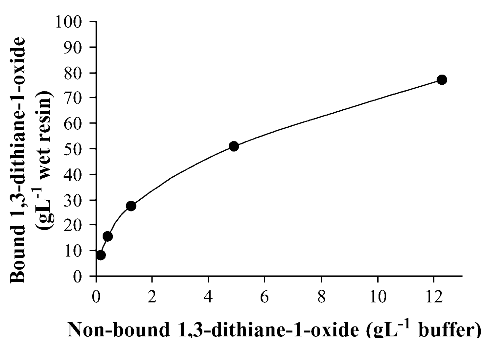


Fig. 3. Binding of 1,3-dithiane-1-oxide on the SP207 resin as a function of non-bound 1,3-dithiane-1-oxide. For conditions, see Section 2.

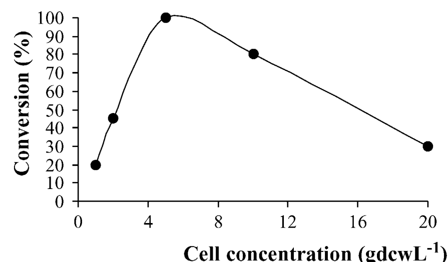


Fig. 4. Rate of 1,3-dithiane (1 g L^{-1}) conversion as a function of cell concentration in shaken-flask experiments.

Table 1
Oxidation of 1,3-dithiane (**1**) catalysed by whole cell of recombinant *E. coli* TOP10 in bioreactor[a]

Entry	Cells (g_{dcw} L^{-1})	Aeration rate (vvm)	Stirring rate (rpm)	**2**		Volumetric productivity ($g\,L^{-1}\,h^{-1}$)
				% Yield	% e.e.	
1	2.5	1.5	400	16	79	0.18
2	5	1.5	700	16	78	0.18
3	5	3	700	12	ND	0.14
4	12	3	700	51	81	0.58
5	5[b]	1.5	400	16	78	0.18
6	5[b]	3	700	12	79	0.14
7	12[b]	3	700	35	80	0.39

[a] Conditions: reaction volume, 1 L; substrate concentration, 5 g L^{-1}; reaction time, 5 h. For details see Section 2.
[b] The resin-adsorbed substrate was used.

shows that the best results were obtained with 12 g_{dcw} L^{-1}, 3 vvm aeration rate, 700 rpm stirring rate, and non-bound substrate (5 g L^{-1}) (Entry 4). In these conditions, the yield of product **2** was of 51% and the volumetric productivity 0.58 g L^{-1} h^{-1}, with an e.e. value of 81%. In the same conditions, but using the resin-adsorbed substrate, the product yield was lower (35%) (Entry 7).

It should be noted that in all cases only small amounts of sulfone **4** and substrate were found at the end of the reaction period (5 h), in spite of the fact that product yield was only in the 12–50% range. These results are due to the high volatility of 1,3-dithiane, as demonstrated by control experiments that showed that, after only 1 h, 30–50% of the substrate was lost, depending on aeration and stirring rates. Thus, the choice of the biotransformation conditions has to be a compromise between the need to avoid oxygen limitation (higher aeration) and minimise loss of substrate (low aeration).

Of course, the use of high concentrations of cells was profitable, because it accelerated the oxidation of the substrate into the less volatile product. Quite unexpected was the finding that the resin-adsorbed substrate, presumably less volatile, did not improve the yield. Potentially, that which can be stripped by the air is the non-bound substrate and since this is always at a low concentration it may be stripped easier. Another possibility is that the rate of substrate release from the resin has become limiting, thereby slowing the reaction, in comparison to non-bound substrate conversions.

It should be noted that the residual cell activity after the biotransformation, determined spectrophotometrically after cell lysis by sonication, was around 80%.

3.4. Biotransformations with isolated CHMO

CHMO catalyses substrate oxidation through a stoichiometric consumption of the expensive co-factor NADPH. Therefore, for preparative applications using the isolated enzyme, it is essential to regenerate NADPH, in situ, at the expenses of a cheap co-substrate by means of a second enzymatic reaction. An efficient co-factor regeneration system is that based on glucose-6-phosphate/G6PDH [18]. However, the use of this system is not economically feasible because of the high cost of the co-substrate. Therefore, in this work the propan-2-ol/ADHTB (alcohol dehydrogenase from *T. brockii*) system was used since it made use of a cheap co-substrate and showed a comparable efficiency.

With the isolated enzyme, it was not possible to use the resin-adsorbed substrate, since the enzyme tended to adsorb on the resin, which caused a strong decrease in catalytic activity. Because of its low aqueous solubility, 1,3-dithiane was present as a solid in the reaction medium when used at concentration higher than 2 g L^{-1}. This solid acted as a reservoir, restoring the substrate in solution as soon as it was transformed; the dissolution was favoured by keeping the reaction under stirring.

The results obtained for oxidation, by isolated CHMO, of different concentrations (4–20 g L^{-1}) of substrate are reported in Table 2. It can be seen that at a concentration of 4 g L^{-1} (Entry 1) all of the substrate was oxidised after 15 h reaction, giving (*R*)-1,3-dithiane-1-oxide **2** in 80% yield and 98% e.e. and the monosulfone **4** in 15% yield. The volumetric productivity of **2** was 0.24 g L^{-1} h^{-1}. At higher substrate concentrations (10 and 20 g L^{-1}), the conversions were still very high, i.e., 95–100%, depending on the conditions (Table 2). However, the enantioselectivities were lower and high e.e. values for product **2** were obtained only at the expense of the yields (Entries 3 and 5). For example, at 20 g L^{-1} of **1**, after 48 h of reaction, the e.e. of product **2** was 97% but the yield was merely 8% (Entry 5).

To explain these results, it must be considered that the optical purity of (*R*)-1,3-dithiane-1-oxide depends both on the asymmetric oxidation of **1** and on the subsequent kinetic resolution of sulfoxides **2** and **3**, since the contaminating (*S*)-sulfoxide is oxidised to the monosulfone **4** faster than the (*R*)-sulfoxide (Scheme 1). The scenario that might be envisaged is as follows. As previously demonstrated, at low substrate concentration (2 g L^{-1}) monosulfone starts to form only after the almost complete consumption of 1,3-dithiane; due to the lower K_m and higher K_{cat} values of CHMO for **1** compared to those for monosulfoxides [23]. However, at the higher substrate concentrations used in this work (10 and 20 g L^{-1}), the situation is different. In fact, while the concentration of solubilised substrate never exceeds 2 g L^{-1} (maximum solubility in aqueous buffer), that of the sulfoxide product (which

Table 2
Oxidation of 1,3-dithiane (**1**) catalysed by isolated CHMO[a]

Entry	Substrate concentration ($g L^{-1}$)	CHMO ($U L^{-1}$)	Reaction time (h)	Conversion (%)	%Yield (%e.e.)		Volumetric productivity ($g L^{-1} h^{-1}$)
					2	**4**	
1	4	1500	15	≥99	80 (98)	15	0.240
2	10	5000	24	96	55 (92)	33	0.260
3	10	5000	48	≥99	6 (98)	80	0.014
4	20	5000	24	95	60 (90)	25	0.570
5	20	5000	48	≥99	8 (97)	76	0.038

[a] The reactions were carried out as described in Section 2, using 10 ml reaction volumes.

is more soluble) is considerably higher. Thus, the oxidation of sulfoxide to sulfone starts well before the complete consumption of substrate **1**, which affects the effectiveness of the process regarding the optical purity of product **2**.

4. Conclusion

CHMO overexpressed in *E. coli* was used in whole cell and isolated enzyme format, to carry out the enantioselective oxidation of 1,3-dithiane (**1**) to the corresponding (*R*)-monosulfoxide (**2**).

Whole cells, which have the advantage of being more convenient and do not need an ancillary NADPH regenerating system, showed a maximum volumetric productivity of $0.18 g L^{-1} h^{-1}$ in shaken flasks (with the resin-adsorbed substrate) and $0.58 g L^{-1} h^{-1}$ in bioreactor (with the non-bound substrate); the e.e. values of the product, 85 and 81%, respectively, were not very satisfactory. The main drawback in the use of the bioreactor which, due to the high aeration, minimises problems of oxygen limitation [31,32], was found to be the high volatility of the substrate which caused severe losses of material. Surprisingly, the approach based on the resin-adsorbed substrate, which has been very successful in other cases [28,29], including the oxidation of bicycloheptenone by whole cells of CHMO [30,32], did not improve the situation.

On the other hand, isolated CHMO gave volumetric productivity values ranging from 0.14 to $0.57 g L^{-1} h^{-1}$. However, only at $4 g L^{-1}$ substrate concentration (Entry 1 of Table 2) was the results really satisfactory (i.e., high e.e. value (98%) and good yield (80%), with a volumetric productivity of $0.24 g L^{-1} h^{-1}$). In the other cases, either the yield or the optical purity of the product was insufficient.

In conclusion, in the case of the synthesis of (*R*)-1,3-dithiane-1-oxide, where the optical purity of the product is of paramount importance, isolated CHMO appears preferable to whole cells, in spite of the fact that the latter biocatalyst form is more convenient.

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