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Correlation between the physicochemical properties of organic solvents and their biocompatibility toward epoxide hydrolase activity in whole-cells of a yeast, *Rhodotorula* sp.

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Abstract

Epoxides are often highly hydrophobic substrates and the presence of an organic co-solvent within an aqueous bioreactor is in such cases indicated. The effect of 40 water-miscible and -immiscible organic solvents on epoxide hydrolase activity in whole-cells of the yeast *Rhodotorula* sp. UOFS Y-0448 was investigated. No formal correlation between solvent biocompatibility and physicochemical properties was deductible, although the introduction of hydroxyl groups increased biocompatibility. 1-Pentanol, 2-methylcyclohexanol and 1-octanol were the most biocompatible resulting in relatively low activity losses when used at up to 20% (v/v).

Introduction

One of the major challenges in conventional aqueous biocatalysis is the low water solubility of hydrophobic substrates that may lead to poor volumetric productivity and downstream processing problems in continuous single-phase aqueous processes (Hari Krishna 2002). It is thus often desirable to have a surfactant or organic phase present in the reaction mixture and the use of purified and semi-purified enzymes in such non-conventional environments has become almost commonplace (Baldascini *et al.* 2001, Hari Krishna 2002). The use of whole-cell biocatalysts in biphasic and non-aqueous media has, however, not yet been explored to quite the same extent.

Water-miscible (hydrophilic) organic solvents have been used as co-solvents in a variety of biocatalytic processes. However, these solvents do not address downstream processing problems and have, in numerous cases, been reported to cause severe enzyme inhibition, even at moderate concentrations (Angelova & Schmauder 1999). The use of water-immiscible (hydrophobic) organic solvents has therefore been considered a more viable option for continuous scale applications. However, as in the case of water-miscible solvents, direct contact between a biocatalyst and an organic layer may result in significant losses in catalytic activity (Choi *et al.* 1999, 2000, Osborne *et al.* 1990), and the main challenge associated with non-conventional aqueous biocatalysis has therefore been the stability of biocatalysts in such environments (León *et al.* 1998).

Several attempts have been made to correlate solvent toxicity with physicochemical parameters such as polarity and molecular weight (Brink & Tramper 1985, Bruce & Daugulis 1991, Laane *et al.* 1985, León *et al.* 1998). With only a few exceptions, the greater majority of such studies have, however, been focussed on toxicity toward bacterial cells, which

leaves a great void regarding whole-cell biocatalysts from other microbial sources, especially yeasts.

Yeast epoxide hydrolases (EHs) are extremely effective in the hydrolytic kinetic resolution (HKR) of chiral epoxides, producing both the residual epoxide and the formed vicinal diol in high optical purities and yields (Orru & Faber 1999). As both enantiomerically pure epoxides and vicinal diols are versatile intermediates in organic synthesis, and as the water solubilities of epoxides are generally low, the development of new and effective methods for the employment of yeast EHs is a focal point in current research. Isolated solvent toxicity studies in yeast cells (Choi *et al.* 1999, Goswami *et al.* 1999) have identified a small number of candidate solvents, but as neither the microbial strains, epoxide substrates, solvents or solvent concentrations of these studies corresponded, the conclusions made were strictly case-related. No general toxicity trend with respect to yeasts, based on the physicochemical properties of the solvents, has therefore yet been deducted.

Materials and methods

General

Rhodotorula sp. UOFS Y-0448 was obtained from the Yeast Culture Collection of the University of the Free State (Bloemfontein, South Africa). Racemic (2,3-epoxypropyl)benzene (EPB) is commercially available and was obtained from Aldrich. Organic solvents from different chemical groups and of varying molecular mass and polarity were chosen on the basis of general availability, and were obtained from Aldrich.

Yeast cultivation

Cultivation was performed in 1000 ml shake-flask cultures containing 200 ml sterilised growth medium, consisting of 0.5% (w/v) yeast extract, 2% (w/v) malt extract, 1% (w/v) peptone and 1.5% (w/v) glucose. After incubation for 48 h at 25 °C, cells were harvested by centrifugation (6000 *g*, 10 min, 4 °C), washed twice with phosphate buffer (50 mM, pH 7.5) and resuspended in the same buffer to 20% (w/v).

Solvent evaluation

Ten ml cell suspension were dispensed into 20 ml glass bottles with screw caps fitted with PTFE/rubber septa. Organic solvents were added to specified final concentrations, after which the bottles were shaken vigorously to ensure adequate contact between the solvents and the cells, and incubated at 25 °C for 10 min. Reactions were started by addition of substrate to give 20 mM, and the mixtures were agitated at 25 °C. Samples (500 μl) were taken at regular intervals, saturated with NaCl and extracted with 250 μl ethyl acetate. After centrifugation (10 000 *g*, 10 min, 4 °C), the organic layers were removed, dried over anhydrous Na_2SO_4 and analysed by chiral GC.

Analysis

Chiral GC was performed using a gas chromatograph equipped with a FID detector and a autosampler-injector, using helium as the carrier gas. Determination of enantiomeric excesses of the residual epoxide and formed diol was achieved by using a β-DEX 225^{TM} chiral fused silica cyclodextrin capillary column (30 m $\times$ 0.25 mm, 0.25 μm film) (supplied by Supelco) at oven temperatures of 110 °C and 150 °C, respectively. Calibration curves for epoxide and diol concentrations were compiled by extraction from reactions employing heat-killed cells in the presence of appropriate amounts of organic solvent.

Results and discussion

The effect of 40 water miscible and immiscible organic solvents on enzyme activity was determined by measuring the initial rate of epoxide hydrolysis (nmol min^{-1} mg dry wt^{-1}) in the presence and absence of 10% (v/v) and 20% (v/v) of each solvent. In attempt to find a correlation between the physicochemical properties and toxicity of the solvents, solvent properties such as molecular weight, density and measures of polarity such as log $P_{octanol}$, polarisability, dielectric constant and dipole moment were correlated to enzyme activity in the presence of the specific solvent (activity expressed as % remaining activity, compared to activity in the absence of solvent).

Log $P_{octanol}$ and pKa values, as well as some of the dielectric constants and polarisabilities not found in literature (Weast 1988, Whim & Johnson 1996), were predicted using ACD/Labs Software^{TM} 4.5 (Advanced Chemical Development Inc., Toronto, Canada), a software package that has been found to be accurate in predicting the physicochemical properties of relatively potential small organic molecules (Hadgraft

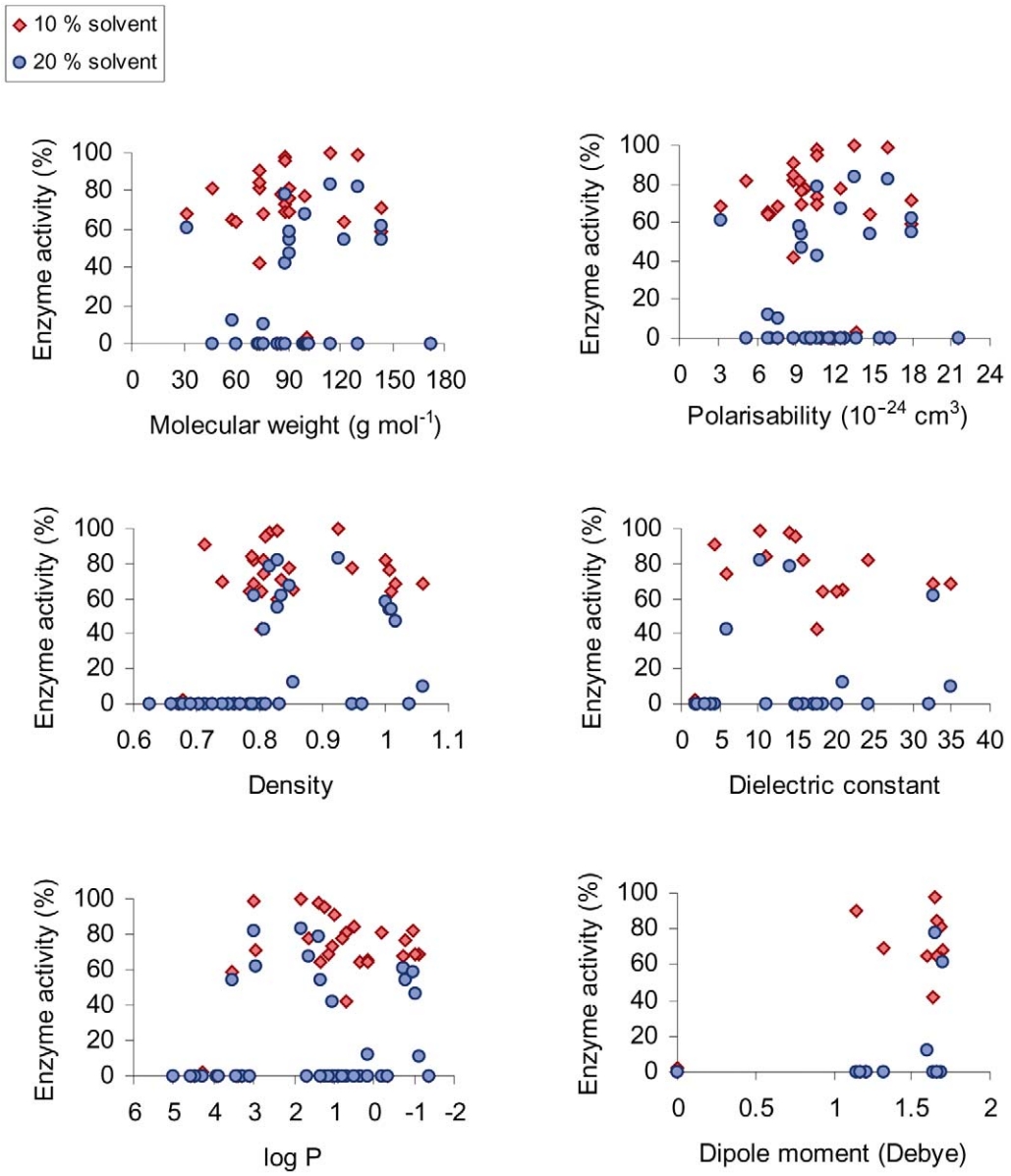

Fig. 1. Enzyme activity versus solvent physicochemical properties.

et al. 2000). Figure 1 shows the potential relationships, between EH-activity in UOFS Y-0448 and the physicochemical properties of the 40 solvents tested. Grouping of the solvents with regard to chemical groups is shown in Figure 2.

From Figure 1 it appears as if there is no direct correlation between enzyme activity and any of the investigated solvent properties. However, grouping of the solvents with regard to chemical groups (Figure 2) reveals a definite increase in biocompatibility with the introduction of hydroxyl groups. From this it is clear that the biocompatibility of the alkanes, alkenes, aldehydes and ethers towards UOFS Y-0448 are, almost without exception, lower than those observed for the alkane diols and mono-alcohols, even at low concentrations.

It was possible to identify three solvents, 1-pentanol (C_5), 2-methylcyclohexanol (C_7) and 1-octanol (C_8), with exceptional biocompatibility at concentrations as high as 20% (v/v), where remain-

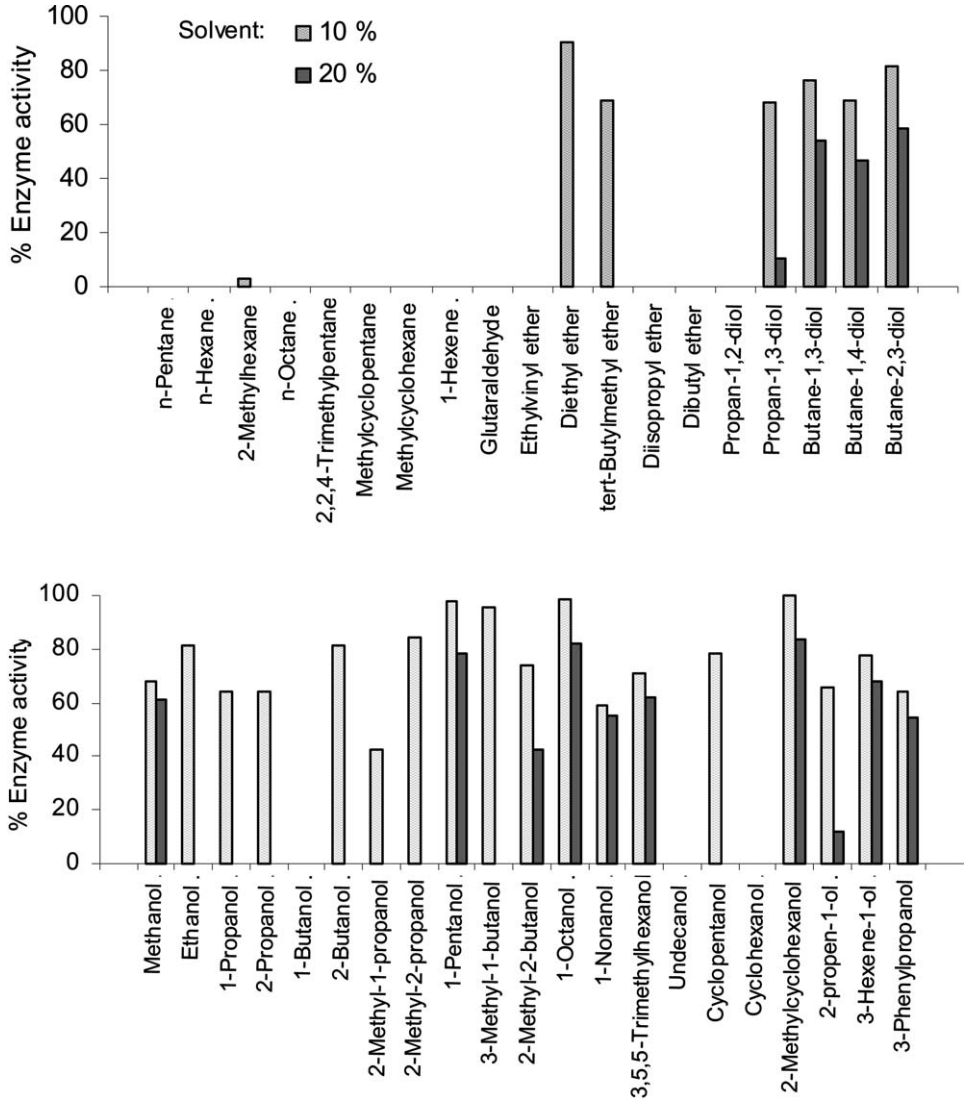

Fig. 2. Grouping of solvents with regard to chemical groups.

ing enzyme activities of 78%, 83% and 82%, respectively, were measured. Consequently, the rates of epoxide hydrolysis in the presence of 5, 10, 20, 30 and 50% (v/v) of each of these solvents were measured and compared to those achieved in the absence of solvent. At low concentrations (5–10% v/v), none of the solvents caused losses of enzyme activity exceeding 2.5%, while moderate solvent concentrations (20% v/v) resulted in activity losses of about 20%. Enzyme inhibition at 30% (v/v) of co-solvent was in all cases more profound, and activity losses of 63% and 78% were calculated for 1-pentanol and 2-methylcyclohexanol, respectively. Inhibition by 30% (v/v) 1-octanol was comparatively low at 33%. High solvent concentrations (50% v/v) resulted in severe enzyme deactivation, with activity losses exceeding 80%, except in the case of 1-octanol, in which an activity loss of 65% was measured.

As these three mono-alcoholic solvents appeared to be reasonably biocompatible toward yeast cells, compared to data previously published on related studies (Choi *et al.* 1999, Goswami *et al.* 1999), a general toxicity trend was searched for within this solvent group. No such trend could, however, be deducted, as some of the alcohols within the C_5–C_8 range [e.g. cyclopentanol (C_5) and cyclohexanol (C_6)] displayed extremely low biocompatibilities. Other alcohols such as 2-methyl-2-butanol (C_5), 1-nonanol (C_9), 3,5,5-

trimethylhexanol (C_9), 3-hexene-1-ol (C_6) and 3-phenylpropanol (C_9) displayed moderate biocompatibilities (which may still appear high when compared to those displayed by solvents from other chemical groups).

Overall, the mono-alcohols as a solvent group, and with special reference to 1-octanol, is at this stage considered most biocompatible towards EH activity in *Rhodotorula* sp. UOFS Y-0448, and may be an appropriate starting point for similar studies on other yeast biocatalysts. However, it is evident that the data thus far gathered with regard to water-immiscible organic solvents is not yet sufficient for accurate predictions regarding solvent toxicity or biocompatibility toward yeast whole cells. Until a comprehensive and accurate database of solvent toxicity trends has been established, the selection of solvents for whole-cell yeast EH applications in biphasic or non-aqueous reaction media will require relevant and well-documented solvent screening procedures.

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