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ORIGINAL PAPER

Predicting enzyme behavior in nonconventional media: correlating nitrilase function with solvent properties

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Abstract The insolubility of nitrile substrates in aqueous reaction mixture decreases the enzymatic reaction rate. We studied the interaction of fourteen water miscible organic solvents with immobilized nitrile hydrolyzing biocatalyst. Correlation of nitrilase function with physico–chemical properties of the solvents has allowed us to predict the enzyme behavior in such non-conventional media. Addition of organic solvent up to a critical concentration leads to an enhancement in reaction rate, however, any further increase beyond the critical concentration in the latter leads to the decrease in catalytic efficiency of the enzyme, probably due to protein denaturation. The solvent dielectric constant (ε) showed a linear correlation with the critical concentration of the solvent used and the extent of nitrile hydrolysis. Unlike alcohols, the reaction rate in case of aprotic solvents could be linearly correlated to solvent log *P*. Further, kinetic analysis confirmed that the affinity of the enzyme for its substrate (K_m) was highly dependent upon the aprotic solvent used. Finally, the prospect of solvent engineering also permitted the control of enzyme enantioselectivity by regulating enantiomer traffic at the active site.

Keywords Solvent engineering · Immobilization · Nitrilase · Enantioselectivity · Enzyme kinetics

Introduction

The use of nitrilases to catalyze the enantioselective synthesis of pharmaceutically important carboxylic acids has attracted attention in the recent years [1, 2]. Search for environmental-friendly and economically feasible alternatives to conventional synthetic methods have been the major thrust behind the development of such technologies. Additionally, the existence of nitrile hydrolyzing enzymes that show *enantio-* and *regio-* selectivity offers synthetic possibilities that are difficult to achieve by conventional catalytic approaches [3]. Inspite of the recognition of their synthetic potential there are only a few successful biocatalytic processes utilizing nitrilases for large-scale production. The major dilemma with nitrile biotransformations is the highly unstable nature of nitrilases [4]. We have earlier shown that entrapment of whole-cell biocatalyst provides a feasible way to enhance its stability and reusability [5, 6]. However, immobilization leads to escalation of mass-transfer limitations which are imposed by the matrix. Moreover, insolubility of the nitrile substrate in aqueous reaction mixture presents an additional challenge to enzymatic nitrile hydrolysis. In this regard, the use of organic solvents to enhance the availability of insoluble nitrile substrate to nitrilase active site has been a very restricted approach, [1, 7] since its behavior in such non-conventional media is not fully understood. Moreover, solvent engineering has been regarded as an appealing alternative to protein engineering [8]. One of the most important industrial application of nitrilases has been the transformation of racemic mandelonitrile to (*R*)–mandelic acid which is catalyzed by several nitrilases from *Alcaligenes* strains (Fig. 1) [9–11]. Recently we explored solvent effects on nitrilase enantioselectivity which allowed us to segregate contributions from activation enthalpy and entropy and elucidating the source of its high enantioselectivity [12]. (*R*)-(-)-mandelic acid is a key intermediate for the production of semi-synthetic cephalosporins [13] and penicillins [14]. It is also used as a chiral resolving agent [15] and chiral synthon for the production

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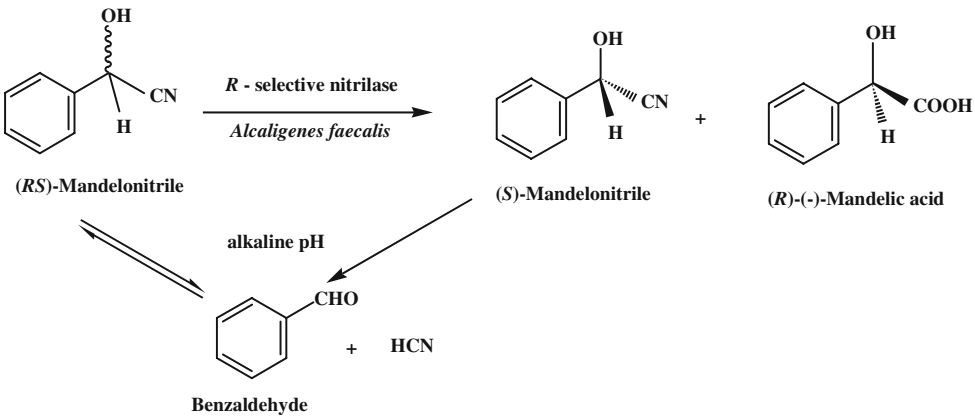

Fig. 1 Scheme for dynamic resolution of mandelonitrile by nitrilase

of anti-tumor [16] and anti-obesity agents [17]. In the present paper an attempt has been made to study the interaction of immobilized whole-cell biocatalyst with various water miscible organic solvents for the production of (*R*)-(-)-mandelic acid. The objective was to overcome the diffusion restriction imposed by the alginate matrix [6], however, no attempt was made to study the effect of mechanical forces on the immobilized preparation.

Broadly, two different classes of solvents were used, alcohols [methanol (MeOH), ethanol (EtOH), isopropyl alcohol (IPA), *n*-propanol (PrOH) and tert-butyl alcohol (*t*-BuOH)] and aprotic solvents [dimethyl sulphoxide (DMSO), tetrahydrofuran (THF), 1,4-dioxan (Dxn), acetone, dimethyl acetamide (DMA), dimethyl formamide (DMF), pyridine, N-methyl pyrrolidone (NMP) and N-vinyl pyrrolidone (NVP)]. Here, we propose some rules for making the choice of a co-solvent and predicting its behavior in water-co-solvent binary mixture based on the correlation of enzyme performance with solvent properties.

Materials and methods

Chemicals

Mandelonitrile, *n*–butyronitrile and sodium alginate were obtained from Sigma-Aldrich Chemical Company (Milawukee, USA.). Growth media components were obtained from Hi-Media Inc. (Mumbai, India). All solvents used were of HPLC grade and other chemicals were of highest purity and were obtained from standard companies.

Microorganism and cultivation conditions

The microorganism, *Alcaligenes faecalis* MTCC 126 was procured from Microbial Type Culture Collection (MTCC, Institute of Microbial Technology, Chandigarh, India) and was grown in a medium of following composition (g/l): ammonium acetate 10, peptone 5, yeast extract 5, dipotassium hydrogen phosphate 5, magnesium sulphate 0.2, ferrous sulphate 0.03, sodium chloride 1 and *n*-butyronitrile 3 (pH 7.2) in a 500 ml shake flask at 30 °C. Cells were harvested after 20 h of induction by centrifugation at 12,000×*g* at 4 °C and washed with buffer before use.

Immobilization of whole cells

For entrapment in alginate, cells were resuspended in the Tris buffer (100 mM, pH 8), added to sodium alginate solution (3%, w/v) and thoroughly mixed to obtain a final concentration of 2.5 mg cells/mg alginate. The mixture was then added drop wise from a syringe to a stirred solution of calcium chloride (2%, w/v). After stirring for two hours, beads were filtered through muslin cloth and stored in fresh calcium chloride solution of same composition until use. Beads were produced with an approximate diameter of 0.18 cm.

Biotransformation conditions

The standard reaction mixture (5 ml) consisted of alginate beads with a total cell loading of 375 mg suspended in Tris buffer (100 mM, pH 8) and appropriate amount of organic solvent (0–40%, v/v). The system was allowed to equilibrate in a rotary shaker (37 °C, 200 rpm) before addition of mandelonitrile (30 mM) solubilized in minimum amount of organic solvent to initiate the reaction. Dielectric constant (ε) values at 20 °C were taken from monographs by Reichardt [18]. The measure of solvent hydrophobicity was the log *P*, where *P* is the partition coefficient of the solvent between octanol and water. The log *P* values were taken from or calculated on the basis of Rekker [19]. The reaction mixture was incubated in rotary shaker (37 °C, 200 rpm) and extent of nitrile hydrolysis was monitored after 8 h and for estimation of initial reaction rate, samples were taken at various time intervals up to 20 min and analyzed by HPLC.

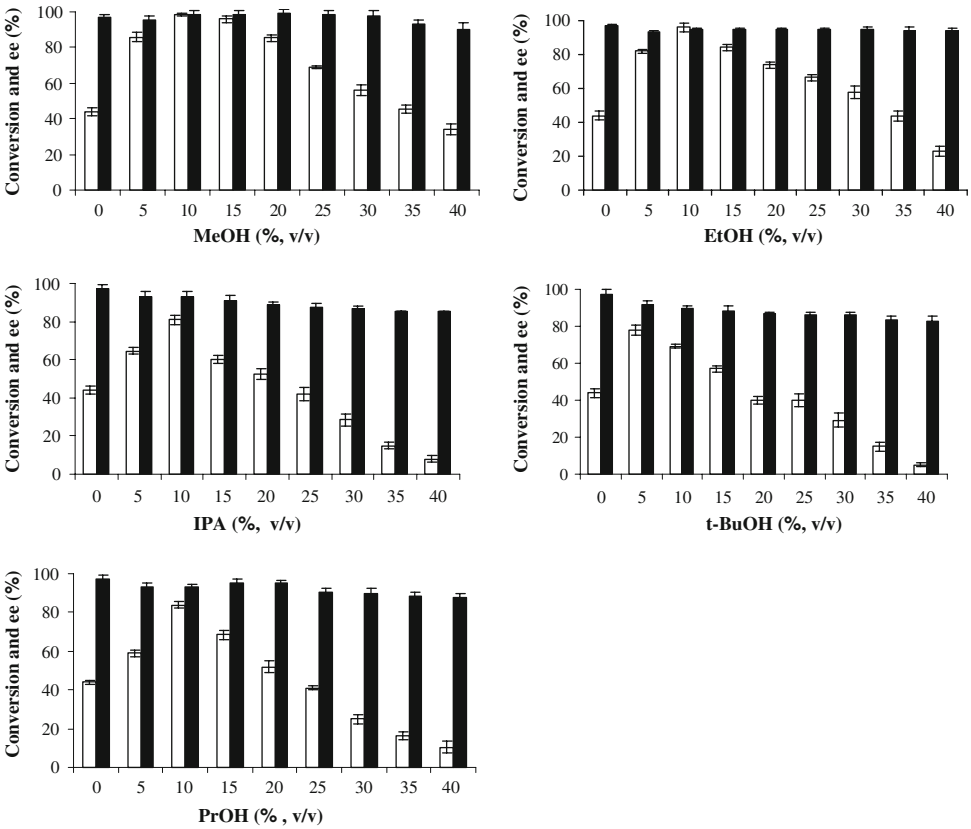

Fig. 2 Conversion and enantiomeric excess (ee) of product in alcohols. *Open bars* represent conversion and *closed bars* represent enantiomeric excess (ee)

Kinetic studies

Kinetic studies were performed by incubating the biocatalyst preparations at varying substrate concentrations and initial reaction rate was measured by taking samples at a regular interval up to 20 min. The product so formed was analyzed by HPLC.

Determination of enantiomeric ratio (*E*)

Preparations of the immobilized biocatalyst were incubated with (R,S)-mandelonitrile in various solvents and the reactions were terminated after different time intervals. For the exact determination, several data points were evaluated at the beginning of the reactions and only data with constant E-values during this time period were used for the calculation.

Isolation of mandelic acid

Cells were removed from the reaction mixture by centrifugation (15,000×*g*, 10 min, 4 °C) and the pH of the reaction mixture was adjusted to 8.5 with 2N NaOH and washed with equal volume of ether. After the pH of the aqueous layer was readjusted to 1.5 with 6N HCl, the desired product was extracted with equal volume of ether. The extract was concentrated under reduced pressure to yield mandelic acid.

Analytical methods

The amounts of mandelic acid and mandelonitrile were assayed by analytical high performance liquid chromatography (model 10AD VP, Shimadzu, Japan) equipped with a LiChroCART® RP-18 column (250 × 4 mm, 5 μm, Merck, Germany) at a flow rate of 0.8 ml/min with a solvent system 0.01 M phosphate buffer (pH 4.8) and MeOH (65 : 35, v/v). The retention times for mandelic acid and mandelonitrile were 3.8 and 20.3 min, respectively. $A_{254\ nm}$ was measured.

The optical purity of mandelic acid was determined by analysis of the enantiomers on CHIRALCEL-OD-H column (250 × 0.46 mm, 5 μm, Daicel Chemical Industries, USA) at a flow rate of 0.5 ml/min with a mobile phase containing hexane, IPA and tri-fluoro acetic acid (90: 10: 0.2, v/v). The retention times for (*S*)-(+) and (*R*)-(-)-isomers were 15.5 and 17.5 min, respectively. $A_{254\ nm}$ was measured.

Results and discussion

Effect of co-solvents on the extent of nitrile hydrolysis

The presence of enzyme in water–organic co-solvent mixture not only affects its catalytic properties but also its stability which may be dependent upon its extent of exposure to the solvent. The extent of nitrile hydrolysis

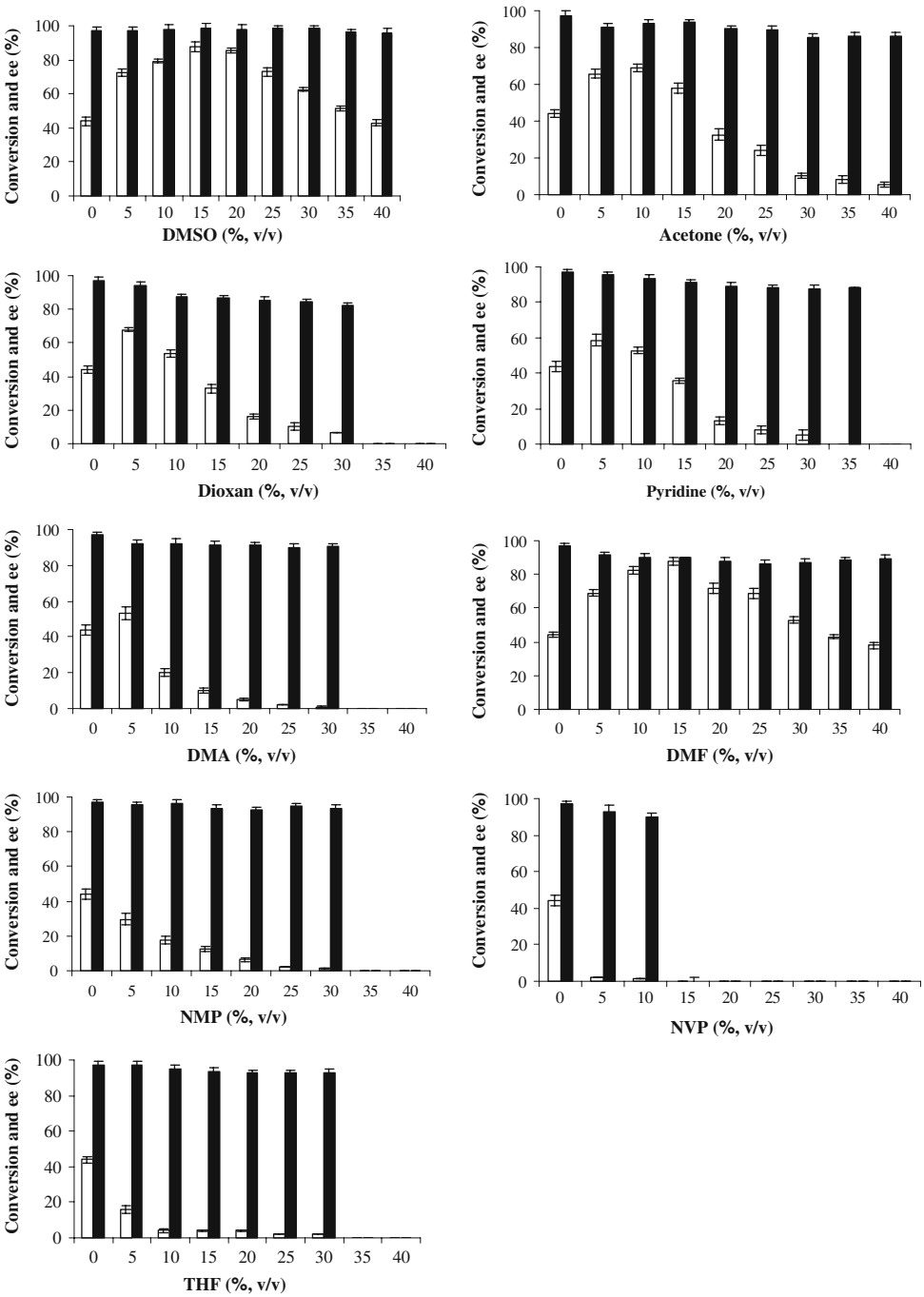

Fig. 3 Conversion and enantiomeric excess of product in aprotic solvents. *Open bars* represent conversion and *closed bars* represent enantiomeric excess (ee)

was significantly enhanced in the presence of alcohols and aprotic solvents specifically in the range of 10–20%, v/v (Figs. 2, 3). It was observed that alcohols were tolerated by the enzyme to a greater extent as compared to aprotic solvents. Highest conversion and optical purity of the product were observed in 10%, (v/v) MeOH and DMF whereas THF was found to result in severe enzyme inactivation. The enantioselectivity of the enzyme remained fairly intact in alcohols, however, in case of aprotic solvents pyridine resulted in significant loss of optical purity of the product.

In biotransformation involving water-organic co-solvent binary mixture, the enzyme performance not only depends upon its conformational flexibility (for activity) but also its structural rigidity (for stability). A critical point is reached when one has to decide the optimal solvent concentration for enhanced enzyme performance without any significant decline in its stability. It is well known that electrostatic forces are one of the factors correlating protein structure and function [20]. Moreover, enzymes have been known to be structurally more flexible in solvents with high ε [21, 22]. Greater structural flexibility will facilitate better

enzyme-substrate interaction for enhanced performance. In this regard, a linear correlation of conversion values was observed with the maximum permissible concentration (MPC) of solvent that can be used without any denaturant effect and the solvent dielectric constant (ε) (Fig. 4a, b). THF, NMP and NVP were not considered for correlation since they caused enzyme inactivation. Among the aprotic solvents, DMA lied outside the correlation range. It may also be noticed that DMA only slightly enhanced the conversion as compared to control (without any solvent) at 5%, v/v, however beyond this range it had a detrimental effect on the enzyme. Alcohols were found to present a more conducive medium for nitrilase function as compared to aprotic solvents, since the enzyme exhibited higher conversion values in the former.

It may further be concluded that solvents with low ε should be added to the medium in low concentration. On the other hand, solvents with high ε may be added in higher concentration without any significant loss of enzyme's catalytic proficiency. The study has therefore, allowed determination of a quantitative criterion for choosing optimum solvent concentration without any deleterious effect to enzyme.

Effect of co-solvents on initial reaction rate

It is well known that an increase in the availability of the substrate to the enzyme active site (EAS) would lead to an enhancement in the reaction rate. In case of alcohols (at 10%, v/v), enzyme exhibited highest reaction rates in MeOH and t-BuOH, however, correlation of the reaction rate with any of the physico–chemical properties of alcohols could not be established (Fig. 5). A linear correlation could be determined between reaction rates and log *P* of aprotic solvents (at 10%, v/v) (Fig. 6).

A stimulatory effect of solvents with high log *P* on the initial reaction rate was expected, based on enhanced solubility and hence availability of substrate around the vicinity of the enzyme. However, the reaction rate was found to reduce as the solvent log *P* increased. To elucidate the possible reason behind this, the effect of solvent log *P* on the reaction kinetics was studied (Fig. 7) and the

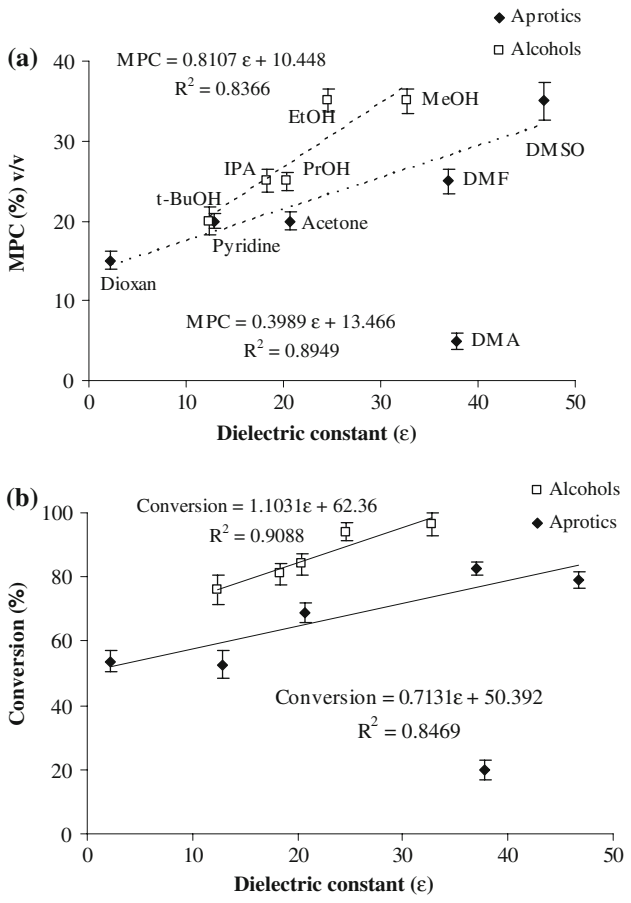

Fig. 4 Correlation of (**a**) MPC and (**b**) conversion values with solvent dielectric constant

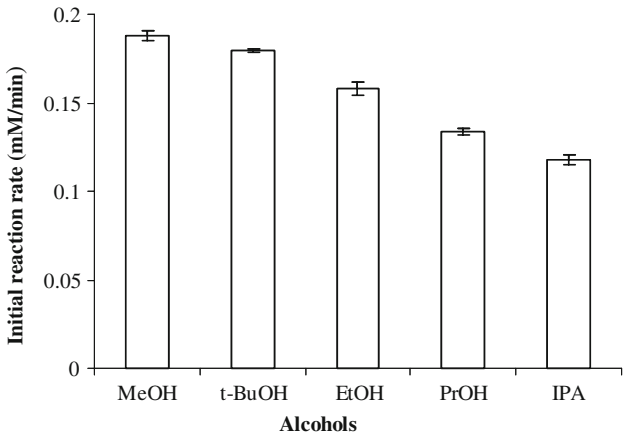

Fig. 5 Initial reaction rates for nitrile hydrolysis in alcohols

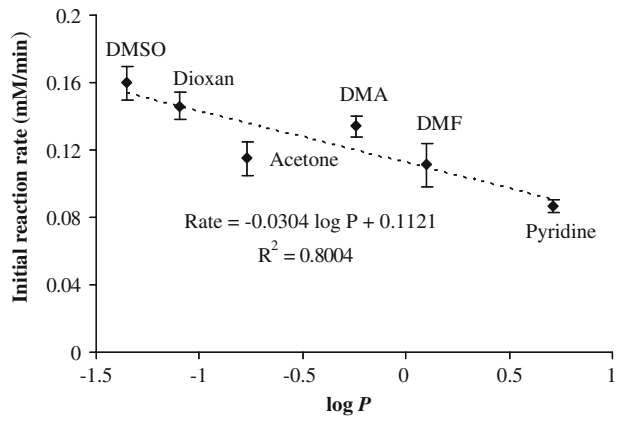

Fig. 6 Correlation of reaction rates in aprotic solvents with log *P* values

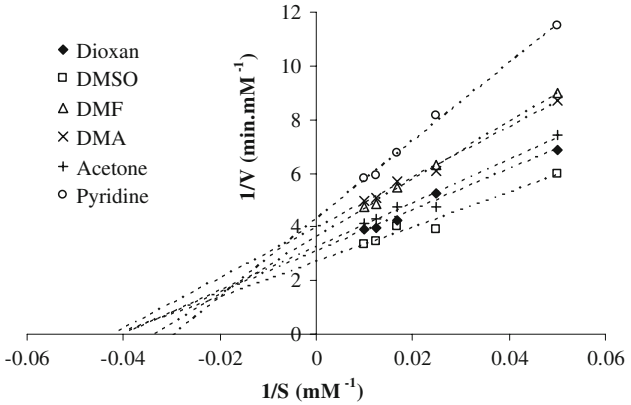

Fig. 7 Lineweaver-Burk plots for estimation of K_m of enzyme in aprotic solvents

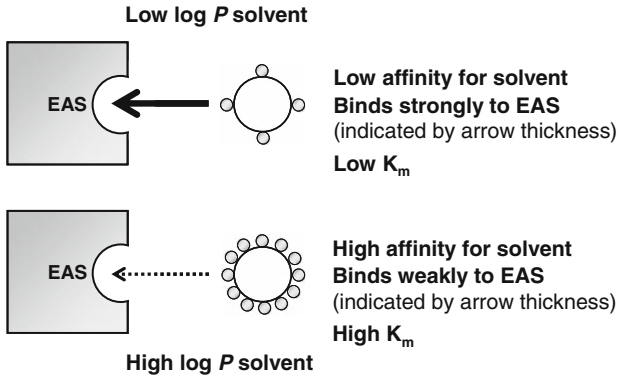

Fig. 9 Schematic representation for explanation of dependence of K_m on solvent log *P*

Michaelis-Menten constants (K_m) were determined (Fig. 8). It may be regarded as the measure of affinity of enzyme for its substrate: higher the $K_{m,}$ lower the affinity and vice-versa. It was observed that the K_m values increased as the solvent log *P* was increased, indicating that the enzyme gradually lost its affinity for mandelonitrile as we moved up the hydrophobicity order. An explanation to this phenomenon could be the rule of 'like dissolves like' (Fig. 9). The first step in the enzyme-catalyzed reaction is the binding of the substrate to the EAS. This involves a desolvation step, in which the substrate molecule is stripped off the solvent molecules attached to its surface. The more strongly the solvent molecules are associated with the substrate, the less favorable will be the binding with the EAS. For the specific case of this reaction, mandelonitrile will be solvated to a greater extent in solvents with higher log *P*. Therefore, the interaction of mandelonitrile with the nitrilase active site will be more favorable in a solvent with low log *P*, resulting in low K_m values and hence greater affinity for the substrate. On the other hand, a far greater amount of energy would be expended in the desolvation step of mandelonitrile in solvents with higher log *P* leading to higher K_m and lower affinity. Another interesting observation was the behavior of enzyme in DMA. In the correlation of MPC and conversion with solvent ε, DMA appeared to lie outside the range; however, in case of correlation of initial reaction rate with solvent log *P*, the data point for DMA was within the limit. This indicates a very complex effect of the extent of exposure of enzyme to the solvent on correlation of its function with solvent properties. When short assay duration (20 min for reaction rate) is chosen, the enzyme is catalytically functional and hence the data point for correlation of reaction rate with DMA, log *P* lies well within the range. On the other hand, longer assay duration (8 h for conversion) sufficiently denatures the enzyme and no correlation could be observed either for MPC or conversion with ε of DMA.

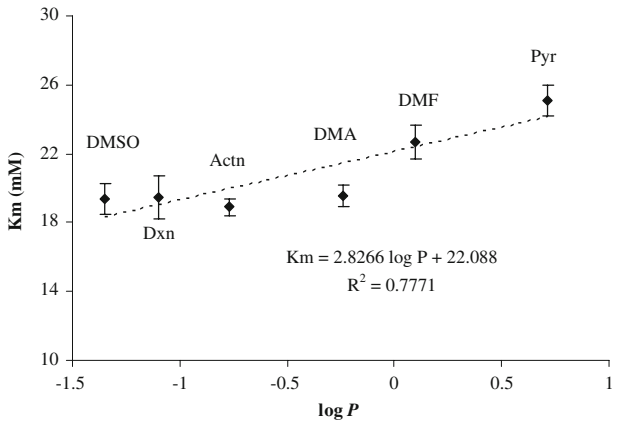

Fig. 8 Correlation of K_m values with log *P* values of aprotic solvents

Effect of co-solvents on enantioselectivity (*E*)

Finally, the effect of various co-solvents (10%, v/v) on enzyme enantioselectivity (*E*) was studied. For the exact determination several data points were considered at the beginning of the reaction and only data with constant *E*-values during this time period were used for the calculation (Fig. 10). DMSO resulted in the enhancement whereas solvents such as Pyridine, DMA and NVP caused severe drop in enantioselectivity. In some cases reversal substrate specificity [23, 24], and even enatiopreference have been reported due to solvent effects [25, 26]. This indicates a very complex interaction of the enzyme with solvents. It may be possible that solvents regulate the kinetics of enantiomer presentation to the EAS to affect its enantioselectivity. In such a scenario, solvents like DMSO may allow selectively the (*R*) enantiomer of the substrate to interact with the EAS and eliminate competition from the slow reacting (*S*) enantiomer, thereby enhancing the 'chiral sightedness' of the enzyme. On the other hand, solvents like Pyridine, DMA and NVP may diminish the ability of the enzyme to discriminate between

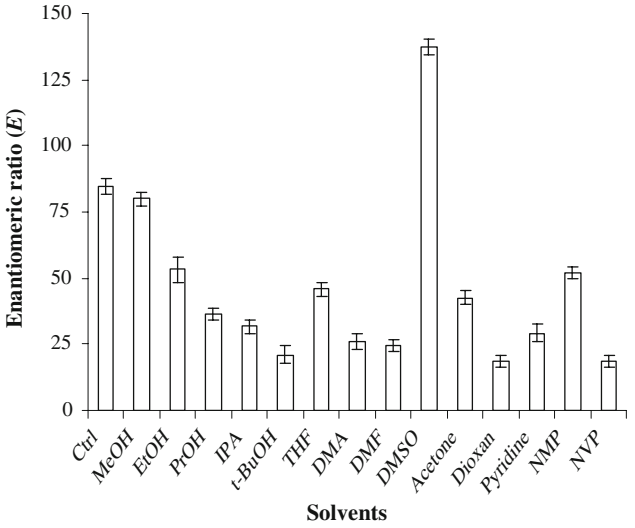

Fig. 10 Effect of cosolvents on enzyme enantioselectivity (E). $E = \ln[1 - c(1 + ee_p)]/\ln[1 - c(1 - ee_p)]$ where c is conversion and ee_p is enantiomeric excess of product

the two substrate enantiomers leading to a state of 'chiral blindness'. It may therefore be regarded that such an approach of solvent engineering offers a means of regulating enantiomer traffic at the EAS.

Conclusion

One of the major problems of biocatalyst immobilization is the exacerbation of mass transfer limitations imposed by the matrix, since a major proportion of the immobilized preparation consists of non-catalytic mass, the matrix. This leads to the decrease in reaction rate and reduced product yield. The insolubility of nitrile substrates in aqueous medium further escalates the mass-transfer problem. We have studied the interaction of immobilized nitrile hydrolyzing biocatalyst with various water miscible organic solvents. The enzyme was best functional in the solvent concentration of 10–20% (v/v). Beyond the critical concentration, the conversion values dropped, probably due to enzyme inactivation upon contact with solvent. Overall, alcohols rated better than aprotic solvents as medium of choice for the reaction. We could obtain a significant correlation between the nitrilase function and different physico–chemical properties of the solvent. This has therefore, allowed us to predict the behavior of the enzyme in such non-conventional media. THF, NMP and NVP caused enzyme inhibition and were not regarded for the correlation. None of the alcohols diminished the optical purity of the product to significant extent as compared to the aprotic solvents. DMSO significantly enhanced the synthetic potential of the enzyme as well as its enantioselectivity.

The study also allowed us to devise a decisive factor for choosing the optimal solvent concentration for enhanced enzyme performance without any harm to the enzyme. The dependence of reaction rate on log P of aprotic solvents was studied on a kinetic level to elucidate how enzymes affinity for its substrate varies with hydrophobicity of solvent. It may also be regarded that extent of nitrile hydrolysis and initial reaction rates were found to be governed by different properties of the same solvent. That is to say, solvent ε had a more global effect on enzyme conformation to control its flexibility and thereby control the extent of conversion; however, solvent log P influenced the interaction between substrate and EAS to control the initial reaction rate. Finally, the approach of solvent engineering also allowed to increase the prospect of regulating enantiomer 'traffic' at the EAS. In this regard, it would be of significant interest to study structural changes induced in the enzyme upon interaction with solvent that regulates the kinetics of presentation of substrate enantiomers to enzyme to affect its enantioselectivity.

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References

1. Banerjee A, Kaul P, Banerjee U (2006) Purification and characterization of an enantioselective arylacetonitrilase from *Pseudomonas putida*. Arch Microbiol 184:407–418
2. Banerjee A, Sharma R, Banerjee U (2002) Nitrile degrading enzymes: current status and future prospects. Appl Microbiol Biotechnol 60:33–44
3. Kobayashi M, Shimizu S (2000) Nitrile hydrolases. Curr Opin Chem Biol 4:95–102
4. Kaul P, Banerjee A, Mayilraj S, Banerjee U (2004) Screening for enantioselective nitrilases: kinetic resolution of racemic mandelonitrile to (*R*)-mandelic acid by new bacterial isolates. Tet Assy 15:207–211
5. Banerjee A, Kaul P, Banerjee UC (2006) Enhancing the catalytic potential of nitrilase from Pseudomonas putida for enantioselective nitrile hydrolysis. Appl Microbiol.Biotechnol 72:77–87
6. Kaul P, Banerjee A, Banerjee U (2006) Enantioselective nitrile hydrolysis by immobilized whole-cell biocatalyst. Biomacromolecules 7:1536–1541
7. Layh N, Willetts A (1988) Enzymatic nitrile hydrolysis in low water systems. Biotechnol Lett 20:329–331
8. Klibanov A (2001) Improving enzymes by using them in organic solvents. Nature 409:241–246
9. Yamamoto K, Oishi K, Fujimatsu I, Komatsu K (1991) Production of *R*-(-)-mandelic acid from mandelonitrile by *Alcaligenes faecalis* ATCC 8750. Appl Environ Microbiol 10:3028–3032
10. Yamamoto K, Fujimatsu I, Komatsu KI (1992) Purification and characterization of the nitrilase from *Alcaligenes faecalis* ATCC 8750 responsible for enantioselective hydrolysis of mandelonitrile. J Ferment Bioeng 73:425–430
11. Liese A (2006) Industrial biotransformations, 2nd edn. Wiley-VCH, Weinheim

12. Kaul P, Stolz A, Banerjee UC (2007) Cross-linked amorphous nitrilase aggregates for enantioselective nitrile hydrolysis. Adv Synth Catal 349:2167–2176
13. Terreni M, Pegani G, Ubiali D, Fernandez R, Mateo C, Guisan JM (2001) Modulation of penicillin acylase properties via immobilization techniques: one-pot chemoenzymatic synthesis of cephamandole from cephalosporin C. Bioorg Med Chem Lett 11:2429–2432
14. Furlenmeier A, Quitt P, Vogler K, Lanz P (1976) 6-Acyl derivatives of aminopenicillanic acid US Patent. Hoffmann-La Roche Inc., Nutley
15. Kinbara K, Sakai K, Hashimoto Y, Nohira H, Saigo K (1996) Design of resolving reagents: *p*-substituted mandelic acids as resolving reagents for 1-arylalkylamines. Tet Assy 7:1539–1542
16. Surivet JP, Vatele JM (1999) Total synthesis of antitumor *goniothalamus* styryllactones. Tetrahedron 55:13011–1328
17. Mills J, Schmiegel KK, Shaw WN (1983) Phenethanolamines, compositions containing the same and method for effecting weight control. Eli Lilly and Company, Greenfield
18. Reichardt C (1988) Solvent and solvent effects in organic chemistry. VCH, New York
19. Rekker R (1977) The hydrophobic fragmental constant. Elsevier, New York
20. Perutz M (1978) Electrostatics effects on protein. Science 201:1187–1191
21. Affleck R, Haynes C, Clark D (1992) Solvent dielectric effects on protein dynamics. Proc Natl Acad Sci USA 89:5167–5170
22. Fitzpatrick P, Klibanov A (1991) How can the solvent affect enzyme enantioselectivity. J Am Chem Soc 113:3166–3171
23. Wescott C, Klibanov A (1993) Solvent variation inverts substrate specificity of an enzyme. J Am Chem Soc 115:1629–1631
24. Zaks A, Klibanov A (1986) Substrate specificity of enzymes in organic solvents vs. water is reversed. J Am Chem Soc 108:2767–2768
25. Wescott C, Klibanov A (1997) Thermodynamic analysis of solvent effect on substrate specificity of lyophilized enzymes suspended in organic media. Biotechnol Bioeng 56:7294–7299
26. Tawaki S, Klibanov A (1992) Inversion of enzyme enantioselectivity mediated by the solvent. J Am Chem Soc 114:1882–1884