Applied biocatalysis beyond just buffers - from aqueous to unconventional media. Options and guidelines.

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Supporting information on solvent systems

Depending on the solvent and water content, different solvent systems are formed. Here the different possible solvent systems are elaborated. Additional examples to what is stated in the main manuscript are provided. The advantages and challenges of the various solvent systems and corresponding catalyst formulations are discussed, with some additional details that could not be accommodated in the main manuscript. A final table in Chapter 4 provides a definitive overview of examples for each reaction system-catalyst formulation combination, should further literature be consulted. More information on different solvent systems and their aspects can be found in the following reviews.1-3

1.1. Aqueous monophasic systems

Buffered solutions, with or without additives, are the most straightforward and commonly used when a novel enzyme reaction is characterized. The lack of a second phase keeps complexity relatively low and enables smooth application to continuous systems.

1.1.1. Pure aqueous reaction phase

As stated in the main text, the application of enzymes in purely aqueous solutions is viable if the reagents are soluble enough to reach on-spec industrial conditions. This is the case for highly soluble substrates like most ions and sugars, for some extremely high-valuable products for which no high product concentrations are required, or for systems where the substrate can be fed over time. A relevant example for pure aqueous enzymatic reactions is the largest commercial industrial biocatalytic application, the use of immobilized D-glucose/xylose isomerase for the production of high fructose corn syrup.4 Other analogous cases are the production of a sweetener, allulose, with 3-epimerase,5 or the early industrial process by DSM, for the conversion of fumaric acid to aspartic acid.6

1.1.2. Cosolvents

Many industrially-used reagents, however, are poorly soluble in aqueous media. Cosolvents, hydrophilic water-miscible compounds, can increase their solubility. In many cases, however, cosolvents are deleterious for enzyme stability, due to disruption of hydrogen bonds provided by water,7-10 which can induce conformational changes in the protein. This effect often starts upward of certain cosolvent concentrations and depends on factors like solvent polarity and the surface.11 Fortunately, proteins can be modified to become more resilient towards cosolvents.12

1.2. Biphasic systems

In enzymatic biphasic solvent systems, a water-immiscible phase is added to the aqueous media. Here, the different possible biphasic systems, depending on the aggregation state and identity of the second phase, are explored in detail. Though the addition of a second reaction phase can be advantageous, it also introduces some complexity. The two main challenges revolve around the interphase and form a dilemma. First, mass transfer over the two phases can limit the reaction if the enzyme catalyzed reaction cannot be sufficiently supplied with reagents. This can generally be countered by an increased surface area, for instance by more vigorous mixing. Unfortunately, most
enzymes tend to deactivate at these interphases.\textsuperscript{13, 14} The increase in surface area is thus generally both beneficial as deleterious for the enzyme catalyzed reaction. The scientific community has recognized these challenges, and various solutions have been proposed, with respect to both reaction and reactor engineering. Reviews and examples have been written on examples on (Pickering) emulsions,\textsuperscript{15, 16} membranes\textsuperscript{17, 18} and flow reactors.\textsuperscript{19, 20} For more information on biphasic systems in biocatalysis in general, several reviews can be consulted.\textsuperscript{2, 21}

1.2.1. Aqueous – Solvent systems

When another solvent (organic solvent, IL, DES) is added to an aqueous buffer, it will, depending on its hydrophobicity, quickly saturate and form a second phase. The reaction components will partition over the two phases according to their partitioning coefficients. Due to continuous exchange of compounds between the two phases – provided that mass transfer limitations are overcome –, the reaction is constantly supplied with substrate while the concentration in the aqueous phase is kept low. Organic solvents are predominantly applied as the second phase, though some examples of immiscible ionic liquids can be found.\textsuperscript{22} The options for the second phase are numerous and the choice will greatly influence the reaction performance. Biphasic solvent systems are feasible independent of biocatalyst formulation, from purified enzymes,\textsuperscript{23-25} to whole cells.\textsuperscript{26-28}

The most intuitive motivation for adding a second phase is to increase substrate loadings of a poorly water soluble compound. However, in case of high partitioning to the organic second phase, concentrations of these components in the aqueous phase can be minimized. This is beneficial if the substrates are toxic or exert an inhibitory effect on the biocatalyst (assuming the biocatalysts to be in the aqueous phase). Similarly, the second phase can be considered as a product sink. For example, for living whole cells, a second phase was introduced to prevent toxicity by the substrates styrene oxide,\textsuperscript{29} 4-vinyl guaiacol\textsuperscript{27} or 1-naphthol.\textsuperscript{30} Another goal of biphasic systems can be to increase product selectivity. Examples can be found for carboxylic acid reduction,\textsuperscript{31} in a cascade of an oxidase and a laccase,\textsuperscript{32} or in a chemo-enzymatic photochemical reaction.\textsuperscript{33} Finally, biphasic systems can also be used to modify reaction equilibria. If the partition coefficient to the second (organic solvent) phase of the product is significantly higher than for the substrate, the concentration of the substrate will stay relatively high in the aqueous phase while the product is being extracted in the organic solvent phase, thereby shifting the equilibrium of the reaction to the desired side. Models on this phenomenon, and a case study with an ADH, were reported by the Greiner group.\textsuperscript{34} Similarly, the equilibrium can be pushed if the product is further converted in the second phase.\textsuperscript{35} Another variant of this principle is reactive extraction, where the product directly reacts with the second solvent. Though this significantly improves thermodynamics, the biocatalyst stability and cross reactivity under these conditions must always be carefully assessed.\textsuperscript{36}

1.2.2. Aqueous – Neat systems

An elegant biphasic variant is the aqueous-neat solvent system, which implies no use of extra solvent. Instead, the substrate concentration in the aqueous reaction is increased beyond saturation, resulting in the formation of a second phase built by the substrate. This option is viable, regardless of the physical state of the substrate. Gaseous, liquid or solid substrates will form gas-liquid, liquid-liquid or slurry reaction systems respectively. Where in the aqueous-solvent system, one has some control on the effective aqueous substrate concentration via the partitioning coefficient, this semi-direct control is more challenging for aqueous-neat systems. If the aqueous substrate solubility needs to be increased, for instance because of a concentration below the Km of the enzyme, water-miscible cosolvents may be supplied by the water phase.\textsuperscript{37} To decrease substrate concentrations is more challenging, though examples for volatile reactants are reported.\textsuperscript{38} As stated above, avoiding the use of extra solvent can be beneficial, both for practical as well as environmental reasons.\textsuperscript{39} It should therefore be assured that
the aqueous concentrations of the substrate do not limit the reaction performance. Furthermore, compared to a liquid monophasic system where the substrate is the only solvent (vide infra), a convenient consequence of aqueous-neat systems is that the total substrate concentration can be controlled by alteration of the ratio of the two phases. One thus has more control over whether full conversion can be achieved, which can avoid cumbersome chromatographic steps with substrate-product separation in DSP.

Examples for gas-liquid systems have been reported for reactions with propane, butane and other volatile substrates, for the fixation of CO₂ via reversed decarboxylation or for reactions with hydrogen and carbon monoxide as electron donors. The most prominent example in literature remains oxygen, which acts as a reactant in many oxidase and oxygenase reactions. A challenge in most gas liquid reactions is the mass transfer limitation, which is often solved with reactor design. To increase mass transfer rates, one can resort to the use of segmented flow, tube in tube reactors or pressured systems. Finally, one should also keep in mind that gas solubility can change from solvent to solvent, if non-aqueous solvents are chosen.

If the substrate is a poorly soluble solid, a slurry reaction system can be applied. This implies that only part of the substrate is solved, while the rest remains solid. Large scale examples are those of the industrial production of Montelukast, or the transformation of solid n-octanoloxime by a aldoxime dehydratase to n-octanenitrile. In both cases, cosolvents were added to to increase substrate concentration in the aqueous phase. The introduction of a solid second phase can also be used to minimize side reactions. For example, soluble sodium sulphite was substituted to its poorly soluble calcium salt to minimize a side-reaction with H₂O₂.

If the biocatalyst substrate is liquid under reaction conditions, the compound can be used in an aqueous - neat solvent system with two liquid phases. Examples are shown for alcohol dehydrogenases, alcohol oxidases, peroxygenases, hydroxynitrile lyases and photodecarboxylase.

1.3. Monophasic non-aqueous systems (Micro-aqueous reaction system (MARS))

The solvent system with the lowest water content presented here is the so-called micro-aqueous reaction system (MARS). The application of MARS most conventionally requires the use of organic solvents, which at first sight seem to compromise the sustainability of the approach. However, MARS typically leads to less solvent waste formation, because of high substrate loadings, and a more straightforward DSP (less work up and possibly less wastewater). In some cases, even increased enzyme stability or changes in selectivity are observed.

Lipases are already commonly used in non-aqueous systems, mostly to drive the reverse reaction in kinetic resolution reactions, and are applied in large scale applications, for example by BASF and Evonik. However, it can seem daunting at first thought to introduce other enzyme classes in a pure solvent system. Especially when it is often observed that at a relative small volume, the solvent already inactivates the enzyme completely. However, when shifting to a pure solvent system, the enzyme activity can be retained in a surprising amount of cases, especially if the enzyme is lyophilized and dispersed in the solvent. Alternatively, the biocatalyst can be immobilized, or expressed in whole cells, which in turn are lyophilized and added to the reaction. Finally, it is worthwhile to include solvent resistance of an enzyme in screening procedures, as this will increase the likelihood that the biocatalyst can be applied in these convenient systems. Altogether, the synergetic impact of optimized process parameters and sustainable process design enabled case studies combining high product titers, simplified downstream processing and promising environmental values.
1.3.1. Neat substrate system

A neat substrate system to be feasible, the substrate needs to meet several requirements, aside from being a liquid under reaction conditions, as not all potential enzyme substrates are eligible. Aldehydes, for example, are very reactive and cannot be added in high concentrations. In this case, the MARS with a feeding strategy results in the highest yields. Nevertheless, when a substrate allows it to be used under these conditions, remarkably productive systems can be obtained. For instance, Erdmann and co-workers showed an approach with the conversion of pure 2-butanone to (R) and (S)-2-butanol in a membrane reactor. Another interesting example is the solid-gas biocatalytic reaction, where the oxidation of gaseous ethanol was catalyzed by immobilized alcohol oxidases.
2. Supporting information on biocatalyst formulation

2.1. Pure enzymes and cell free extract

Free enzymes generally display high activities and specific space-time yields, acting as a homogeneous catalyst. They often reside in the aqueous phase of biphasic systems, with the exceptions of several hydrolases, which often reside in the organic phase or at the interphase. Biocatalysts from other classes are often more challenging to use as purified enzyme in mostly hydrophobic systems, though examples with decent activity in various concentrations of organic solvents exist.

2.2. Immobilized purified enzymes

Enzyme immobilization can be considered as a tool to increase enzyme stability, e.g. towards non-aqueous solvents or other reaction conditions, or to increase handling of the enzyme, e.g. to enable reusability or to retain the enzyme in a continuous reactor. Numerous methods of immobilization have been reported, with different goals in mind.

Two general ways to immobilize enzymes are inclusion/encapsulation/entrapment and coupling. The preferred option depends on the requirements and the system. For example, a stable coupling method is often chosen specifically for reusability or retention in continuous systems, while an important criterion for inclusion may be to provide additional protection for enzymes from harsh or incompatible reaction conditions. In both cases, but especially when entrapped, mass transfer limitations can be introduced. Especially the entrapment of (purified) enzymes into hydrogels is an option to apply enzymes in reaction systems with high concentrations of hydrophobic substrates. As an example, Begemann and co-workers combined an ADH with a formate dehydrogenase for cofactor recycling in a hydrogel in a continuous biphasic system with integrated pH control. The benefits were also shown in several studies by Ansorge-Schumacher and co-workers for lipases and ADHs. For peroxygenases, both encapsulation in hydrogels and covalent binding on beads enabled application under neat conditions.

Immobilization of enzymes increases the cost of the catalyst and thus has to result in higher product titers and/or more enzyme turnovers, to make the process economically feasible. One frequently used option for process intensification is the use of continuous reactors to achieve this goal. Continuous reactors with a neat substrate system can amount to very productive systems, when the covalently bound enzyme is efficiently retained during the reaction.

2.3. Whole cells

Whole cells as biocatalysts show similar advantages as purified enzymes but come at lower costs. But they are only applicable is background activity does not negatively interfere with the targeted biotransformation. Here, the catalysts are either living (resting or viable), or lyophilized cells. The application of living organisms can be advantageous as the catalyst will multiply over the reaction course, further decreasing catalyst cost. Furthermore, the simultaneous introduction of complex synthetic pathways is enabled. However, most organisms are susceptible to organic solvents as these can interact with the lipid membrane, which leads to growth inhibition, metabolic damages or even cell death. One solution to this challenge is to work with solvent-tolerant microorganism strains, like members of the genus Pseudomonas. This enables synthetic and systems biologists to work under extraordinarily (aromatic) substrate or solvent conditions without lethal effects to the microorganism. A pioneer in this direction is the group around Schmidt and Bühler, which deals with effects on the viability of the microorganisms, product concentrations and purity, sustainability, suitable solvent selection and much more. It was shown that, compared to the conventional E. coli production strain, Pseudomonas taiwanensis was more viable for the production of styrene oxides. A switch away from E. coli as a production host, however, also means one forfeits all the expertise, toolboxes and previous
engineering on this accustomed organism. Fortunately, effort is put to also customize *Pseudomonas* variants for this purpose.\(^{101-103}\)

Where viable cells are susceptible to organic solvents, lyophilized whole cells are more conveniently used in non-conventional media.\(^{72}\) Lyophilization affects the bacterial cell in many ways, depending largely on the procedure, the strain, and potential additives. In general, bacterial cells are damaged by the procedure, which predominantly result in cell death.\(^{104}\) This can impair them from growth in this formulation after rehydration, however the enzymatic activity is usually retained. However, some cells might still be viable (therefore viability must be explicitly excluded if no GMO license is available). More inactivation effort may be required here. Compared to free enzymes, the shell around the enzyme, which increases stability of the entrapped enzyme and fosters re-usability, comes already with the produced enzyme for free. Furthermore, the tedious and expensive enzyme purification as well as additional immobilization is not necessary, which decreases production cost of the catalyst in comparison to a purified enzyme by 90 %.\(^{89}\) Examples of the use of lyophilized cells are shown for the production of epoxides\(^{29, 105}\) and poly-ε-caprolactone in biphasic systems.\(^{17, 106}\)

Though the application of whole cells does bring advantages with respect to costs and simplicity, one also introduces the native reactions of the cell to the system. All organisms harbour their own enzymes to keep their metabolism running, which are introduced to the system with the cells. Reaction selectivity should therefore be monitored if this formulation is chosen.

### 2.4. Immobilized whole cells

Though whole cells suspended in the reaction mixture are conveniently separated from the reaction mixture during downstream processing (DSP), the catalyst is harder to retain in continuous systems, such as in flow. For this purpose, immobilization of whole cells can not only increase catalyst re-usability and thus decrease cost,\(^ {107}\) but also be a powerful tool to stabilize biocatalytic activity. It can also prevent enzymes from e.g. lyophilized whole cells from leaking into the aqueous phase. Recent projects e.g. address the coating of whole yeast cells to provide compatible conditions for chemo-enzymatic cascades by protecting the microgel coated cells from inactivation in the presence of copper catalyst.\(^ {108}\) Whole cells can be retained with membranes, for example in so-called teabags filled with catalyst,\(^ {109}\) or be stimulated to form biofilms.\(^ {110}\) This can be exploited in reactors, where high cell-densities and a high retention of biomass was combined with high productivities in tubular photobioreactors.\(^ {111, 112}\) Biofilms are a special form of immobilized biocatalyst, which provides remarkable resilience towards toxic substances, which can be exploited for the conversion of non-natural, harmful substances. Examples for this are the oxygenation of cyclohexane\(^ {38}\) or styrene\(^ {113}\) using *Pseudomonas* strains or the dehalogenation of haloalkanes using *E. coli*-derived enzymes in *P. putida*.\(^ {114}\) As with free whole cells, one should be aware of possible side reactions.
3. Supporting information on solvents

3.1. Solvent options

3.1.1. Organic solvents

The most commonly used solvents to increase the solubility of hydrophobic compounds in biocatalysis are organic solvents. The amount of organic solvents available are numerous, each with a different influence on enzyme stability and activity, and downstream processing. From a practical point of view, the polarity of the solvent is often the first parameter considered, typically assessed with the Log P. Solvents with a Log P close to zero or lower will be water miscible and can thus be applied as cosolvents. High Log P solvents, on the other hand, are only sparingly soluble and will quickly form a second layer. Though solvent polarity is a good starting point in solvent selection, the performance of organic solvents in media is also, amongst others, influenced by their hydrogen donating or accepting abilities and molar volumes.115

3.1.2. Ionic liquids and deep eutectic solvents

A common alternative to organic solvents for biocatalytic reactions are ionic liquids (IL). ILs typically consist of anion and cation pairs that only bind loosely to each other and are thus liquid at room temperature. Advantageously, as an ionic solvent, these liquids are able to solve compounds of widely different polarities in one mixture.116 Furthermore, their non-volatility and non-flammability make them attractive from a safety point of view. Their non-volatility also makes product recovery relatively convenient.117 Unfortunately, IL synthesis can be step intensive, associated with toxicological issues, and the batches might suffer from variations due to the tedious production process.118, 119 For these reasons, an emerging popular alternative for these conventional ILs are deep eutectic solvents (DES). DESs are made from halide salts and hydrogen bond donors and share typical beneficiary properties of ILs like non-volatility and flammability and low melting point. On top of that, DESs are relatively straightforward to synthesize from renewable components while also being biodegradable. From a green chemistry point of view, DESs are thus preferred over classical ILs.

Just as organic solvents, ILs and DESs can be applied as miscible cosolvents, immiscible solvent in biphasic systems or pure as the reaction mixture itself.120 In some cases, one can even switch in between one- and two-phasic systems by modulating the reaction temperature,121, 122 Next to increasing the solubility of many reaction compounds, the application of ILs and DESs can also lead to an increase of biocatalyst stability and selectivity.123-125 Furthermore, through astute design, the solvent can also act as a reactant, as a dual function solvent, as was shown for glucose or glycerol in DESs.126-129 The properties of ILs are defined by several parameters, like polarity, size, and place in the Hofmeister series. Considering the effect on enzyme stability, some coherent correlations have been found in accordance with the latter.130

3.1.3. Supercritical solvents

A final option worth mentioning here are the supercritical fluids, gasses which are between a liquid and a gaseous state at certain pressures and temperatures. These solvents are especially interesting as their solubilizing abilities can be conveniently altered by changing the reaction’s temperature and/or pressure,131 which provides a great advantage in product recovery. The most used example in biocatalysis is supercritical CO\textsubscript{2} (scCO\textsubscript{2}) due to its moderate critical point (31.1 °C and 7.38 MPa). Its abundancy, non-flammability and non-toxicity cause that scCO\textsubscript{2} is generally considered to be a “green” solvent. Reports show that scCO\textsubscript{2} efficiently solves other gasses and olefins and that its superfluidity enables high diffusion rates.132, 133 Furthermore, in biphasic systems, scCO\textsubscript{2} can also be used in combination with other non-conventional solvents.134 One restriction in scCO\textsubscript{2} as a solvent is its limited use in combination with living cells, as it is toxic towards most microorganisms.135 Furthermore, the
requirement for specialized pressured equipment prevents its general use in common labs. In some cases, non-critical gasses can also serve as a solvent, as was shown in work by the group of Spiess and Büchs with immobilized ADH\textsuperscript{136-138} and earlier in work by Klibanov.\textsuperscript{74} This application, however, is limited to volatile substrates.

3.2. Solvent choice per system

The choice of a (co)solvent for biocatalytic reactions depends, ultimately, on several aspects. First, a solvent should appropriately solve the reagents in question, while also enabling adequate work-up. Secondly, it must be not too deleterious to enzyme activity and stability, as this may compromise the overall synthetic procedure. Finally, aspects on the sustainability of the solvent should be taken into consideration.

3.2.1. Which solvent to choose as cosolvent?

Low Log P (polar) organic solvents are water miscible and commonly used as cosolvents, though the use of ILs and DESs as cosolvents are gaining importance as well. Typical organic cosolvents that reoccur in literature are DMSO, acetonitrile, or low molecular weight alcohols (methanol, ethanol, isopropanol or tert-butanol).\textsuperscript{139} As their only role is generally to aid solubility, the cosolvents are ought not to take part in any (side-) reactions with the substrates or products. An exception are dual function solvents, which act both as cosolvent and cosubstrate. In case of cofactor regeneration, compounds like acetone (for oxidative regeneration) or isopropanol (for reductive regeneration) can act both as cosubstrate and cosolvent to reduce side-product formation. Multiple examples for different systems can be found in the following examples.\textsuperscript{37, 69, 105, 140, 141} Often, adding the cosubstrate as cosolvent is also beneficial for thermodynamic reasons, as the excess can drive the reaction to the desired side.

The addition of cosolvents can affect the activity and selectivity of enzymes, even at low concentrations. A wide distribution of these effects, depending on cosolvent-biocatalyst combinations, is shown in the works by Gerhards et al., on carboligase reactions\textsuperscript{142} and Schumacher et al., on an alcohol dehydrogenase reaction.\textsuperscript{143} In the case of viable whole cell systems, cosolvents can positively influence reaction rates by increasing cell wall permeability. This has been shown for both organic solvents\textsuperscript{144} and ILs.\textsuperscript{145} Unfortunately, the addition of cosolvents, in most cases, has a detrimental effect on protein stability.\textsuperscript{146} Whole cell systems are generally found to be more resistant to cosolvents, as compared to free enzymes in solution.\textsuperscript{69} For example, in the work of Müller et al., up to 60% of a DES could be added to a whole cell ADH reaction without loss of activity. Under similar conditions, the free enzyme was quickly deactivated.\textsuperscript{123} Addition of cosolvents to a biocatalytic reaction can thus have widely different effects on the enzyme behaviour, depending on the enzyme-solvent combination. As these parameters are numerous and intertwined, we do not have the means yet to accurately predict the effect a cosolvent will have. But modelling tools are appropriate which might facilitate planning in the nearer future.\textsuperscript{147} It is therefore advised to always dedicate some experimental work to optimize the biocatalytic reaction.

3.2.2. Which solvent to choose for biphasic systems?

For biphasic liquid systems, organic solvents are the most common choice, though through specific combinations of ions, buffer and temperature, ILs can also act as a second phase.\textsuperscript{120} The first thing to consider when designing a biphasic system is the partitioning coefficient of the compounds in the formed medium, as this will determine their concentrations in both solvents. High partitioning coefficients are not always beneficial, as one should also consider the affinity of the biocatalyst to the substrate. If the Km of the enzyme is relatively high, predominant partitioning of the substrate to the second phase (in which no catalyst is present) might decrease the concentration in the aqueous phase as such that Kcat turnover frequencies are not achieved.\textsuperscript{148, 149} An example is the work by Leuchs et al. who performed the reduction of aliphatic ketones by an ADH. The team saw a significant decrease in reaction rate for
compounds with longer chain lengths, as the increase in hydrophobicity caused them to partition more to the organic phase.\textsuperscript{149}

Partitioning of an apolar solute does not necessarily scale with Log P of the solvent,\textsuperscript{150,151} but rather improves if the Log P of the solvent and solute are similar. Hydrogen accepting and donating abilities, polarizability, the Hildebrand solubility parameter and the molecular volume of the solvents further influence partitioning.\textsuperscript{152} The volume ratio of the two solvents also influences partitioning,\textsuperscript{25,153,154} as a larger organic phase fraction will generally benefit partitioning towards the organic phase. However, this also increases the volume of organic solvent in the reaction, which can have a negative effect on DSP.\textsuperscript{30} Several models to give an indication of the partition coefficient of reactants in biphasic systems have been developed. One example is COSMO-RS,\textsuperscript{152,155} developed by Klamt \textit{et al.},\textsuperscript{196,157} which includes quantum chemical calculations on charge densities and chemical potentials. These predictions make initial suggestions for solvents possible, which reduces the amount of required experimental work.\textsuperscript{158,159} To gain more insight for a specific reaction setup, the partition coefficient can be determined experimentally.\textsuperscript{160}

Aside from partitioning behaviour of the reaction compounds, biocatalyst stability is paramount. When a solvent forms a second phase, some of it will dissolve in the aqueous phase and \textit{vice versa}, which can have a deleterious effect on the catalyst. To minimize this effect, apolar, water immiscible solvents are often preferred.\textsuperscript{161} As mentioned earlier, sensitivity towards organic solvents is also observed for whole cell systems with living cells.\textsuperscript{22,145} The toxicity of solvents with a Log P below 1 is relatively low to microorganisms. However, solvents with a Log P between 1 and 5 are able to cross the cell wall and accumulate in the membrane space.\textsuperscript{162} This can disrupt the integrity of the cell wall, meaning loss of functionality and energy production. In the end, the presence can cause membrane-leakages, resulting in cell-death. Some organisms are more resistant to these effects than others are. This is mainly determined by membrane composition.\textsuperscript{94,163} ILs in biphasic systems have been reported to have less detrimental effect on the cell membrane.\textsuperscript{22}

Finally, the choice of organic solvent can be restricted by the reaction itself. An obvious example is if the solvent possesses functional groups that can be accessed by the enzyme. The conventional solvent group of ketones,\textsuperscript{2} for instance, best be avoided in ADH or transaminase reactions to avoid unwanted side-product formation.\textsuperscript{148} Furthermore, specific inhibition of the enzyme by the solvent should be assessed. As examples, longer aliphatic alcohols were reported to inhibit the activity of an amine oxidase.\textsuperscript{164} Toluene completely inhibited the activity of an aryl alcohol oxidase,\textsuperscript{57} supposedly due to \textpi-\textpi stacking with the flavin cofactor.\textsuperscript{165}

Concluding, the choice of solvent in a biphasic system may influence the reaction performance of several different levels, which makes predictions unreliable. It is therefore advised to select several, preferably benign, solvents of varying Log P in a solvent screen. The optimal solvent choice in terms of DSP does not necessarily reflect the optimum for biotransformation. Here, a compromise between effective DSP and transformation must be found or a suitable operating window for both aspects must be defined.

3.2.3. Which solvent to choose in micro aqueous reaction systems?

The solvent selection in a micro-aqueous reaction system follows the same basic rules with respect to enzyme stability and reaction design as discussed for biphasic systems. First and foremost, the solvent should not interfere with the reaction or (strongly) inactivate the enzyme. The key difference between MARS and biphasic solvent systems is the low amount of water present in MARS, only up to saturating concentrations (a few percent in volume to volume). The (non-bulk) water mainly encloses the biocatalyst, forming a hydration shell around the enzyme or around/inside the whole cell catalyst, which in turn is surrounded by the solvent. For MARS, mainly apolar solvents are applied, as polar solvents can strip away this hydration shell, which results in enzyme deactivation.\textsuperscript{1} The mentioned discrepancy in ratio of the water and solvent is especially important concerning the partitioning coefficients of all compounds in the reaction. For instance, if the substrate has a relatively low Log P value, its concentration in the hydration shell will be relatively high. This results in a higher local substrate concentration, which can lead to decrease enzyme activity in case of substrate surplus inhibition.
Likewise, very apolar products tend to be present in the apolar solvent thus shifting the equilibrium to the product side. As a consequence, the solvent choice can also affect the equilibrium of the reaction.\textsuperscript{166}

The effect of solvents on the activity of the commonly used lipase B from \textit{Candida antarctica} are addressed in a thorough review by Kumar and co-workers.\textsuperscript{62} Aside from lipases, only a limited amount of studies have screened solvents in MARS for other enzymes, e.g. oxidoreductases or thiamine diphosphate dependent enzymes. Jakobinnert and co-workers found little correlation between the Log P value and the respective enzyme activity was observed. The only general observation was that low Log P solvents generally show lower conversion. Furthermore, the addition of small quantities of highly concentrated buffer (up to 1 M) caused higher enzyme activity than the addition of water alone.\textsuperscript{67} Still, the optimal buffer species and amount was, as so often in biocatalysis, enzyme specific. Similar observations were made in a later manuscript on this topic.\textsuperscript{167} To provide more accurate prediction in the use of solvent in MARS, more studies are required, though conventional solvents like MTBE and, the more “green” option, cyclopentyl methyl ether (CPME) show promising results. Next to organic solvents, DESs have been used under micro-aqueous conditions, both with DES as the main solvent\textsuperscript{168} and with the substrates forming a DES in a neat system.\textsuperscript{169}
4. Overview formulations and solvent systems

Table S1. Overview of examples from literature for combinations of enzyme formulations and solvent systems for several enzyme classes.

| Enzyme formulation | Aqueous, monophasic | Biphasic | Non-aqueous, monophasic |
|--------------------|---------------------|----------|------------------------|
| Free enzyme        |                     |          |                        |
|                    | Oxidoreductases⁴⁹, ¹⁷⁰ | Oxidoreductases⁵⁷, ⁵⁹, ¹⁴⁸, ¹⁷⁷ | Oxidoreductases⁶⁹ |
|                    | Hydrolases¹⁷¹, ¹⁷² | Hydrolases¹⁷⁸ | Hydrolases¹¹⁵, ¹⁸² |
|                    | Lyases¹⁷³, ¹⁷⁴     | Lyases¹⁵³, ¹⁷⁹, ¹⁸⁰ | Transferases¹⁶¹ |
|                    | Transferases¹⁷⁵, ¹⁷⁶ | Transferases¹⁸¹ |                        |
| Immobilized enzyme |                     |          |                        |
|                    | Oxidoreductases⁷⁷, ¹⁸⁴, ¹⁸⁵ | Oxidoreductases⁷⁷, ¹⁸⁹, ¹⁹⁰ | Oxidoreductases⁸⁷, ¹⁴⁰, ¹⁹³, ¹⁹⁴ |
|                    | Hydrolases⁷⁶, ¹⁸⁶ | Hydrolases¹⁹¹ | Hydrolases⁷⁶, ¹⁹⁵, ¹⁹⁶ |
|                    | Lyases¹⁸⁷          | Lyases¹⁷³, ¹⁹² | Transferases¹⁹⁷ |
|                    | Transferases¹⁸⁸    |                        |                        |
| Whole cells        |                     |          |                        |
|                    | Oxidoreductases¹⁹⁸, ²⁰⁰ | Oxidoreductases³⁰, ¹⁹⁹, ²⁰⁶ | Oxidoreductases³, ⁷⁵, ²⁰⁰ |
|                    | Hydrolases²⁰¹, ²⁰² | Hydrolases²⁰⁷, ²⁰⁸ | Lyases⁶⁸ |
|                    | Lyases²⁰³, ²⁰⁴     | Lyases²⁷, ²⁰⁴ | Transferases²¹⁰ |
|                    | Transferases²⁰⁵    | Transferases²⁰⁶, ²⁰⁹ |                        |
| Immobilized whole cells |                  |          |                        |
|                    | Oxidoreductases²¹¹, ²¹² | Oxidoreductases³⁰, ¹⁴⁵ | Oxidoreductases²¹⁸ |
|                    | Hydrolases²¹³     | Hydrolases²¹⁵, ²¹⁶ | Lyases²¹⁹ |
|                    | Lyases²⁰⁴, ²¹⁴     | Lyases²⁰⁴ |                        |
|                    | Transferases²¹¹    | Transferases²¹⁷ |                        |
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