Automated Enzyme Screening Methods for the Preparation of Enantiopure Pharmaceutical Intermediates

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Received: November 12, 2002; Accepted: January 25, 2003

Supporting information for this article is available on the WWW under http://asc.wiley-vch.de or from the author.

Abstract: In a pharmaceutical environment, the need for efficient and practical screening techniques has become vital in the search for ideal enzymes that can be used in the preparation of drug intermediates. This paper describes a general high throughput screening (HTS) protocol that has been validated by a number of global projects within Pfizer. It outlines the procedures for the preparation of screening kits, as well as protocols for reaction set-up, optimization and analysis. The need for such protocols in routinely evaluating the use of solvent engineering in enzymatic hydrolysis reactions is also outlined, with several examples provided. The advantages and disadvantages of a number of complementary analytical tools that are being used in the analysis of enzymatic reactions are also discussed.

Keywords: biocatalysis; enzyme catalysis; high throughput screening; hydrolysis; kinetic resolution

Introduction

Throughout the last two decade, biocatalysts have been increasingly used in the preparation of drug intermediates and metabolites.[1] In the synthesis of chiral compounds, enzymes have emerged as very attractive alternatives to conventional chemical methods.[2] The use of this technology, however, is still limited partly due to the lack of an efficient and practical high throughput screening (HTS) process that is required to identify the desired biocatalysts from hundreds of potential enzymes as well as a number of potential reaction parameters under which they can optimally function.[3] The fact that such parameters, including solvent, solvent content, pH, temperature, time, and cosubstrates, are many times correlated non-linearly makes the screening task even more challenging. There have been no general efficient screening protocols reported in the literature. The more traditional screening practice is to use a limited number of enzymes under a limited number of reaction parameters, and this often results in the failure to identify many potential enzyme hits.

The need for an efficient HTS protocol becomes even more necessary when one begins to routinely evaluate the use of medium or solvent engineering in enzymatic reactions. Several examples discussing the use of medium engineering have been reported in the last 20 years,[4,5,6,7] and two scenarios are generally discussed: enzymes suspended in organic solvents for acylation-type reactions and those in aqueous media for hydrolytic reactions. The former scenario has been well documented in the literature,[6,8] and it is generally accepted that lipases can exhibit dramatic changes in enantioselectivity when switching from one solvent to another. When optimizing hydrolysis reactions, however, an additional variable comes into play: *the solvent content*. Therefore, a thorough solvent screen should involve careful analysis of both solvent type and solvent composition. When applied in our laboratory, an HTS approach has produced a high success rate in identifying and tuning selective enzymes. Furthermore, the use of solvents, when compared to other approaches used to produce "designer enzymes" *via* mutation and recombinant DNA technology, is faster, less expensive and scaleable.

This paper will describe a general HTS protocol that has been validated by a number of global projects within Pfizer. Using this approach, hundreds of reactions can be set up and analyzed within days, which otherwise might require weeks using simple vial preparation of reactions. A great advantage gained by using this protocol is that it requires very small amounts of both the enzymes, which may be quite expensive, as well as the racemic substrates being screened, which are commonly pharmaceutical intermediates available in limited amounts. The fundamental basis behind this HTS protocol is that enzymes can be stabilized and stored for months in suitable 96-well plates under certain preparative conditions. A second essential

  1615-4150/03/34504-524 – 532 $ 17.50-.50/0

requirement is that a number of different instruments be made available to analyze a variety of substrates screened *via* these 96-well plates. The combination of this screening protocol with statistical optimization algorithms can quickly and efficiently allow the user to predict important conditions and suggest where further optimization should proceed.

Results and Discussion

Preparation of Screening Kit

The general HTS screening approach is illustrated in Figure 1. The first step **(**Figure 1, step 1) involves the one time preparation and storage of screening kits. This step usually takes on the order of 1 – 2 days and entails the preparation of the enzyme stock solutions (100 mg/mL) and the dispensing of these stock solutions into 96-well screening plates, or "screening kits". The dispensing of the stock solutions into a screening plate is carried out by an automated liquid handler workstation, which can be programmed to accurately dispense 10 μL of each individual enzyme into the appropriate location in each 96-well plate. This system has been used to prepare hundreds of screening kits and has saved valuable time and effort.

Screening kits have been organized according to two screening levels. A 'Level 1′ screening kit (see Figure 2) consists of less expensive commercially available hydrolases, including lipases, proteases, acylases, esterases and amidases, that can potentially be practical in a large scale process. During the screening process, a 'Level 1′ screening kit is always screened first. A 'Level 2′ screening kit, consisting of more expensive hydrolases such as those engineered *via* directed evolution, is used only if the first kit fails to yield any desired enzymes. The identity of the enzymes found in the first level screening kit is included in the supplementary materials section.

When choosing a 96-well plate format, the plates need to be both solvent- and temperature-resistant, therefore, it is recommended to use polypropylene plates. The plate volume and morphology also need to be considered. The reactions herein are carried out in 100 μL reaction volumes in plates no larger than 500 μL.

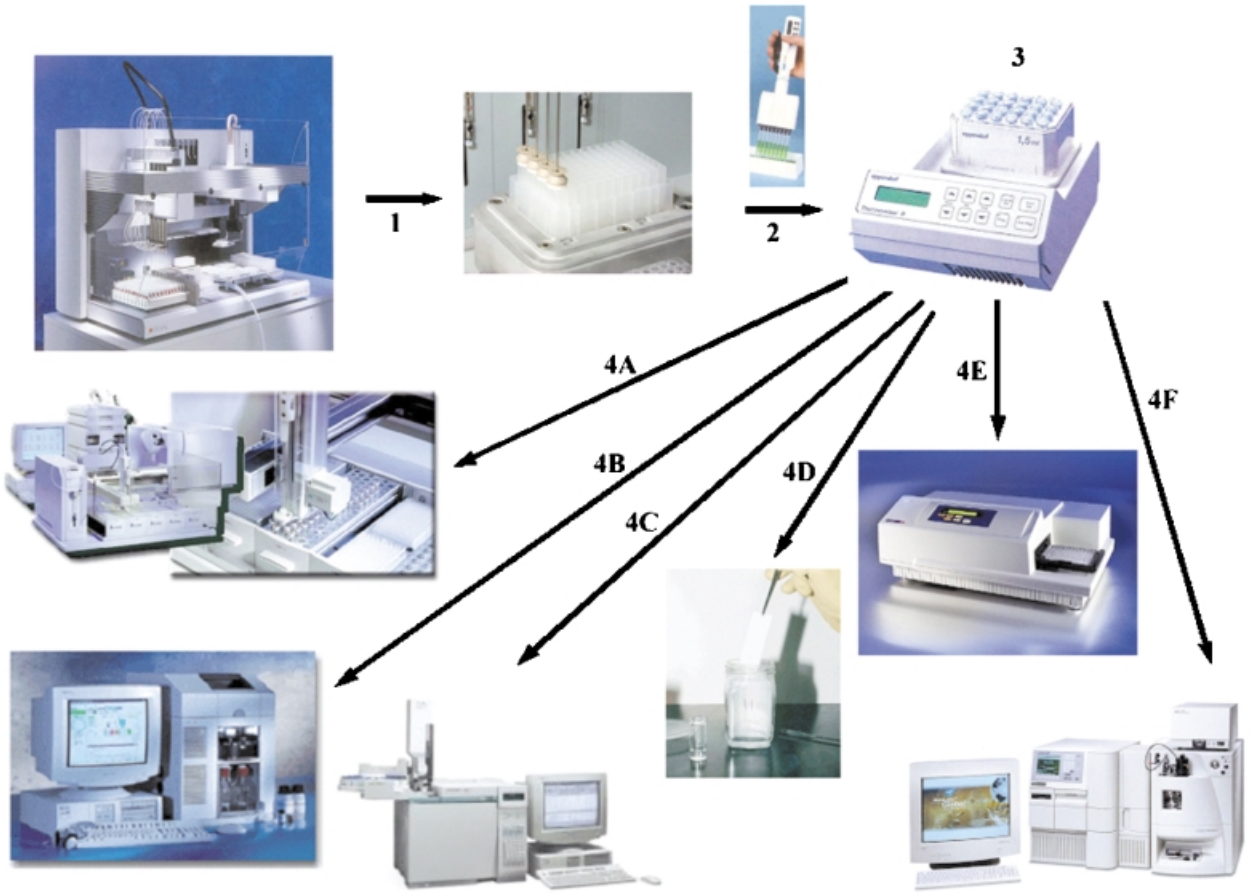


Figure 1. Summary of HTS approach. Step **1** consists of the one-time automated preparation and storage of enzyme screening kits. Step **2** entails the 5 minute preparation of 96 enzymatic reactions with a desired substrate. Step **3** entails the incubation of reactions. Step **4** entails reaction analysis: **4A** – HPLC; **4B** – CE; **4C** – GC; **4D** – TLC; **4E** – UV/Vis; **4F** – LC/MS.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PGRD1[a]	PGRD9	PGRD17	PGRD25	PGRD33	PGRD41	PGRD49	PGRD57	PGRD65	PGRD73	PGRD81	PGRD89
B	PGRD2[b]	PGRD10	PGRD18	PGRD26	PGRD34	PGRD42	PGRD50	PGRD58	PGRD66	PGRD74	PGRD82	PGRD90
C	PGRD3	PGRD11	PGRD19	PGRD27	PGRD35	PGRD43	PGRD51	PGRD59	PGRD67	PGRD75	PGRD83	PGRD91
D	PGRD4	PGRD12	PGRD20	PGRD28	PGRD36	PGRD44	PGRD52	PGRD60	PGRD68	PGRD76	PGRD84	PGRD92
E	PGRD5	PGRD13	PGRD21	PGRD29	PGRD37	PGRD45	PGRD53	PGRD61	PGRD69	PGRD77	PGRD85	PGRD93
F	PGRD6	PGRD14	PGRD22	PGRD30	PGRD38	PGRD46	PGRD54	PGRD62	PGRD70	PGRD78	PGRD86	PGRD94
G	PGRD7	PGRD15	PGRD23	PGRD31	PGRD39	PGRD47	PGRD55	PGRD63	PGRD71	PGRD79	PGRD87	PGRD95
H	PGRD8	PGRD16	PGRD24	PGRD32	PGRD40	PGRD48	PGRD56	PGRD64	PGRD72	PGRD80	PGRD88	PGRD96

[a] Control A (1 – 38 Lipase) (39 – 81 Protease) (82 – 96 Others)
[b] Control B

Figure 2. Example illustrating the typical format of a HTS enzyme screening kit. A similar format is adopted for both Level 1 and Level 2 screening kits. Enzymes are organized by Pfizer Global Research and Development (PGRD) number. Note that location A1 and B1 are usually reserved for control reactions (usually consisting of substrate incubated in buffer in the absence of enzyme). For detailed information on the identity of enzymes in level 1 screening kits, please see supporting information.

V-bottom plates tend to improve solution agitation and allow for cleaner sampling after centrifugation.

Once the plates are prepared, they can be sealed with either adhesive foil or with a penetrable mat cover and stored at $-80\ ^{\circ}C$ for months or even years. By preparing hundreds of screening kits and freezing them only once prior to use, the time needed in thawing the entire master stock plate and pipetting the enzyme solutions into the reaction plate is saved, and the activity of the enzyme solutions is much better preserved because only one freeze-thaw cycle is necessary. Validation studies were conducted to prove the viability of the enzymes after one freeze-thaw cycle and are illustrated in Figure 3. As can be seen from the chart, the residual activities for most of the enzymes after one freeze-thaw cycle are $>90\%$ of their original activity prior to freezing and thawing. These validation studies are conducted on a monthly basis, and it has been shown that the reactivity is not diminished even after one year of storage under these conditions. The similar reactivity profiles observed from month to month also demonstrate the reproducibility exhibited by the use of these plates.

In cases where anhydrous conditions are required, such as acylation and amidation reactions, the screening plates must be lyophilized prior to use. In order to remove the water from each well, the plate is lyophilized for 10 to 24 hours at a chamber temperature of $-20\ ^{\circ}C$. After lyophilization, the plates can be stored at 4 to $8\ ^{\circ}C$. Similar validation studies as those shown in Figure 3 for the residual activity of the enzymes after one freeze-lyophilization cycle were also performed. The majority of enzymes retained $>85\%$ of their activity as compared to the value prior to freezing and lyophilization.

Reaction Set-Up

After the initial preparation and storage of a large number of screening kits, running a reaction plate is accomplished with relative ease (Figure 1, step 2).

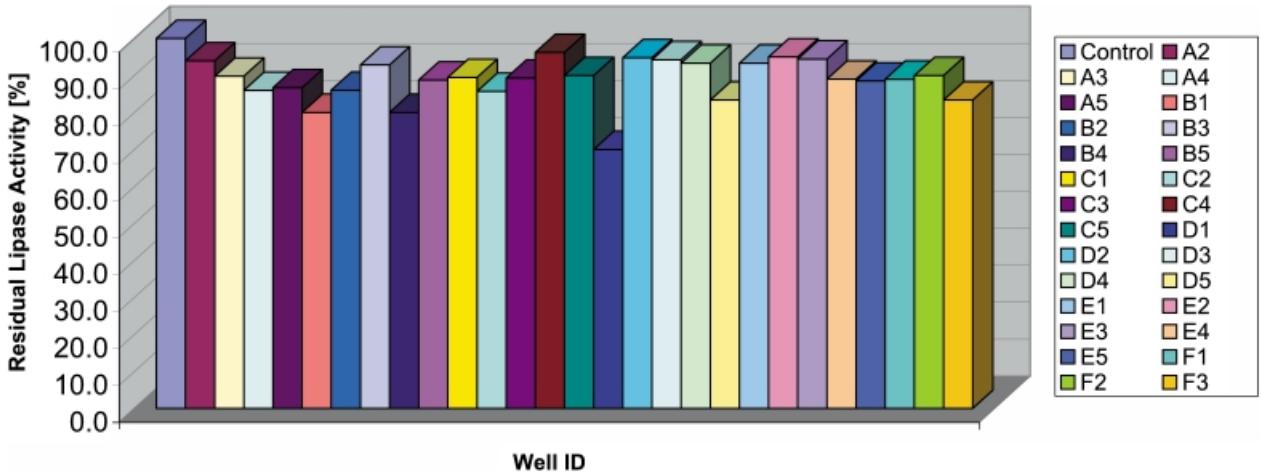


Figure 3. Residual activity of enzymes after one freeze-thaw cycle. Activity was determined *via* Sigma Lipase PS assay. Residual activity was calculated according to the following formula: Residual Activity = activity of enzyme after one freeze-thaw (or freeze lyophilization cycle)/activity of enzyme prior to any freeze-thaw (or freeze lyophilization) cycles $\times$ 100.

Generally, reactions are conducted on a 100 μL scale, with final reaction conditions in each well consisting of 1 mg/mL of substrate, 10 mg/mL of enzyme, 10% organic solvent, and a final pH of 7.2. The first step in performing a screen is to prepare the substrate stock solution at a concentration of 10 mg/mL. Ideally, the types of hydrolases that are being screened should be taken into consideration when developing the conditions of the reaction. For example, lipases exhibit interfacial activation, and hence screening should ideally be carried out using water immiscible cosolvents such as MTBE, which would result in a biphasic screening system.[9] Furthermore, most lipases are optimally active at a neutral pH. Proteases, on the other hand, should be screened using a water miscible cosolvent such as acetonitrile and the optimal pH can vary according to the type. Esterases, acylases and amidases should be screened using a water miscible co-solvent and a neutral pH. Table 1 outlines a range of solvents that can be used during the screening and optimization stages of reactions. Solvents are subdivided into two major classes: water-miscible and water-immiscible. For each class, solvents are arranged in order of hydrophobicity value, determined by calculating their octanol-water partition coefficients.[10] When running large numbers of reactions during the screening stage, there is a balance that needs to be reached between optimal screening conditions and issues of practicality. In a pharmaceutical environment, that balance most often falls on the side of practicality, therefore at the screening stage, a quick universal screening protocol is employed for all hydrolases. Although adopting this methodology might result in a potential hit not being fully recognized, this minor risk is compensated by the time saved.

The screening procedure takes on the order of 10 minutes for each substrate. First, the screening kit is allowed to thaw for 5 minutes. 80 μL of potassium phosphate buffer (0.1 M, pH 7.2) are pipetted into the wells *via* a multichannel pipette, and the plate is agitated for 5 minutes in an incubator/shaker. 10 μL of the substrate stock solution, which is prepared at 10 mg substrate/mL organic solvent, are then added to each well, again *via* a multichannel pipette, and the 96 reactions are incubated at a constant temperature and agitation rate in an incubator/shaker (Figure 1, Step 3). The recommended default conditions are 30 °C and 750 rpm. Using this procedure, only 10 mg of substrate are required to screen 100 reactions.

Sampling 96-Well Plate Reactions

If necessary, the 100 μL reactions can be sampled multiple times. Samples with a volume of 25 μL are removed from the reaction mixture at the desired time points *via* a multichannel pipette and are dispensed into a new 96-well plate. Reaction samples are then quenched by the addition of 150 μL of acetonitrile. The protein, in most cases, will precipitate out upon the addition of acetonitrile, and the 96-well plate is centrifuged to allow the precipitate to pellet at the bottom of the V-shaped wells. The organic supernatant is then extracted from each well into another 96-well plate. In case multiple plates need to be sampled, the liquid handler can easily be set up to perform the operations quickly and efficiently. The sampling procedure is implemented to ensure that analytical instruments and chromatography columns are not damaged by the presence of proteins. An alternative although more expensive approach that has been used more recently is to simply filter the quenched samples *via* a 96-well filter plate. Sampled reactions are then sealed using a penetrable mat cover and transferred to the appropriate instrument for analysis.

Table 1. Solvents used during screening and optimization. Solvents are subdivided into 2 main categories: water-miscible and water-immiscible.

Solvent Code	Water-Miscible Solvent	Log P[a] value	Solvent Code	Water-Immiscible Solvent	Log P Value
M1	DMSO	– 1.35	I1	hexane	4.00
M2	DMF	– 1.01	I2	cyclohexane	3.44
M3	methanol	– 0.74	I3	toluene	2.73
M4	acetonitrile	– 0.34	I4	1-hexanol	2.03
M5	ethanol	– 0.30	I5	chloroform	1.97
M6	acetone	– 0.24	I6	dichloroethane	1.86
M7	2-propanol	0.05	I7	DIPE	1.52
M8	*tert*-butyl alcohol	0.35	I8	dichloromethane	1.25
M9	THF	0.46	I9	*tert*-pentyl alcohol	1.28
M10	1,4-dioxane	NA	I10	MIBK	1.31
M11	acetic acid (5%)	NA	I11	MTBE	0.94
M12	TEA (5%)	NA	I12	ethyl acetate	0.73
M13	pyridine (5%)	NA			

[a] Log P values correspond to octanol-water partition coefficients (adapted from Sangster[10]).

Reaction Analysis

As shown in Figure 1 (step 4), some of the analytical tools available include high performance liquid chromatography (HPLC), capillary electrophoresis (CE), gas chromatography (GC), UV spectrophotometry, and liquid chromatography coupled with mass spectrometry (LC-MS). Choosing which analytical tool to use depends on the nature of the substrate, mainly its absorbance properties and volatility. Reactivity of the enzyme toward the substrates is usually analyzed first, followed by the analysis of the reactive hits using chiral methods. The same sampled 96-well plate can be used to analyze both reactivity and enantioselectivity.

The most commonly used instrument is the automated 96-well plate HPLC system (Figure 1, 4A). The main criterion for substrates to be suitable for HPLC analysis is that they must contain a chromophore with an absorbance range from 200 to 300 nm. HPLC is ideal for quantitative reactivity screening, where a variety of 3 minute methods have been developed and used to separate $>95\%$ of the reactants from their corresponding products. As a consequence of a short analysis time, quantitative results for 96 reactions can be attained in less than five hours. HPLC is also heavily used for analysis of the enantiomeric excess in each sample. However, despite the high success rate in finding adequate HPLC methods for chiral separation, the analysis time is the main drawback, ranging on the order of 10 – 50 minutes per sample. When dealing with 30 or 40 enzyme hits, followed by detailed optimization studies, the reaction analysis time can become overwhelming.

The progress made in capillary array electrophoresis (CE) technology has resulted in partial circumvention of this issue (Figure 1, 4B). Reetz et al. recently outlined the use of CE to achieve 'super – high-throughput screening' for enantioselective catalysts.[11] As compared to HPLC, analysis time for CE ranges on the order of only 3 – 10 minutes. Parallel 96-well high throughput analysis has recently become available, decreasing analysis time by at least two orders of magnitude. However, these instruments are still quite expensive and require special maintenance. The criteria for substrates to be suitable for CE are the same as those for HPLC, an adequate method of separation and a chromophore in the substrate. It is important to note, however, that despite its advantages, CE is by no means a replacement for HPLC. CE has not been universally successful in separating all enantiomers that were resolved *via* HPLC, and as a result, the two technologies complement each other.

Gas chromatography (GC), based on an FID detector technology, is mainly used for compounds that do not contain a strong chromophore and as such, do not absorb light in the UV range (Figure 1, 4C). GC is especially useful for the detection of simple chiral amines and alcohols. For these compounds, some chemical derivatization might be needed to increase volatility. A toolbox of chiral GC columns should cover more than 90% of racemic amines and alcohols. Method development is also quite simple for GC as the only variables that need to be manipulated are the temperature ramp and choice of column. Most GC systems can also be easily outfitted with 96-well plate autosamplers. Analysis times range on the order of ≤ 5 minutes for reactivity and are usually ≤ 15 minutes for enantioselectivity.

A crude yet effective method of analysis is thin layer chromatography (TLC) (Figure 1, 4D). This technique can provide a quick and qualitative reactivity screen. Commercially available 8-channel TLC spotters can significantly enhance the analysis process for 96-well plates. TLC is very powerful in that analysis times are usually very short. Chiral TLC plates can also be used to determine whether the enzyme is enantioselective towards the substrate, however this method is qualitative and in most cases, quantitative data are more desirable.

Some groups have exploited the use of pH indicators as a simple method to follow ester hydrolysis, whereby the release of the carboxylic acid product results in a drop in pH[12,13] (Figure 1, 4E). Kazlauskas and coworkers have implemented the "Quick E" method, which has been shown to give relatively accurate measurements of E values in most cases. Despite its efficiency, this method is still considered an indirect approach to measure enantiomeric excess and also requires substrates that are available in their enantiomerically pure forms.

Mass spectrometry can be a very powerful technique to check for the enantioselectivity of reactions (Figure 1, 4F). Reetz and coworkers have followed the kinetic resolution of isotopically labelled pseudo-enantiomeric substrates by mass spectroscopy.[14] This technology is practical in cases where hundreds to thousands of identical screens are anticipated and the enantiomers are readily available in their enantiomerically pure forms. In a pharmaceutical environment, however, time and high turnover of substrates are major factors which must be considered, and the bottom line is that the isolated enantiomers of most intermediates that require screening are not readily available, especially for discovery projects, where the chiral synthesis is not pursued in the early search for potential drug leads. Despite this, preparative chiral chromatography can be used to provide small quantities of pure enantiomers that can later be screened *via* mass spectrometry or methods such as "Quick E".

Other techniques which can also be used in the measurement of enantiomeric excess include circular dichroism (CD) and fluorescence. After applying the experience gained in using all of the above-mentioned techniques, however, the following conclusion has been

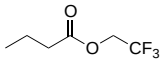
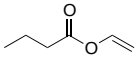
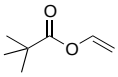
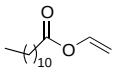
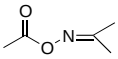
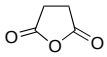
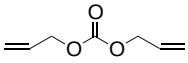
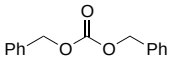
reached: in a pharmaceutical environment, screening for a desired biocatalyst required for a particular application is best achieved by monitoring the reaction of interest rather than relying on surrogate substrates. Therefore, focus has centered on the development of efficient analytical methods such as HPLC, CE, and GC that can provide direct quantitative results. Moreover, these three analytical tools are complementary and are required to support screening for a wide range of potential intermediates and substrates.

Influence of Solvents on Hydrolytic Kinetic Resolutions

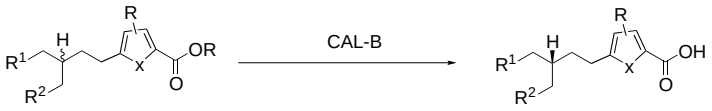
In our experience, 25 to 33% of the enzymes screened will be active towards the substrate, and of those, only 10 to 15% might be enantioselective. If the initial screen uncovers even a mildly enantioselective enzyme, there exists the potential to dramatically improve that enantioselectivity by conducting a comprehensive solvent screen using solvents such as those listed in Table 1. The preparation of these types of reactions can be done manually or can be automated using a liquid handler workstation. In many cases, however, the substrate being screened is quite precious, and a simple and economical procedure is followed to ensure that a minimal amount of substrate is necessary and that substrate loading is consistent. First, a substrate solution at 10 mg/mL is prepared using a volatile solvent such as acetone or acetonitrile. The substrate solution (10 μL) is then dispensed into each well of a clean 96-well plate *via* a multichannel pipette. The solvent is then evaporated using a rotary concentrator, leaving consistent quantities of dry substrate in each well. The appropriate solvents and solvent quantities are then added to each well. The various enzymes are then added to the plate by diluting the enzymes in the prepared screening kit with a certain amount of buffer and transferring the enzyme solutions to the plate with substrate. Once the enzymatic and solvent screens are completed, further optimization can be accomplished using a combination of experimental data and statistical design programs.

In the case of optimizing acylation reactions, two main parameters are investigated in order to tune the reactivity and enantioselectivity of an individual enzyme. First, solvents are screened using one acylating agent to identify the ideal solvents that exhibit the potential for selectivity. After this is accomplished, various acylating agents are screened against the ideal solvents in order to identify the conditions that will further optimize the reaction. A list of common acylating agents, organized in order of their reactivity, is provided in Table 2. If the above trials fail to yield a sufficient process, one can attempt to examine other means of enzyme activation by the addition of excipients prior to lyophilization of the enzyme.[15]

Table 2. Acylating agents used during screening and optimization. Acylating agents are illustrated in order of reactivity, with the most reactive agents first.

Label	Name	Structure
A1	2,2,2-trifluoroethyl butyrate	
A2	vinyl acetate	
A3	*n*-butyric acid vinyl ester	
A4	pivalic acid vinyl ester	
A5	lauric acid vinyl ester	
A6	isopropenyl acetate	
A7	acetyl acetone oxime	
A8	succinic anhydride	
A9	diallyl carbonate	
A10	dibenzyl carbonate	

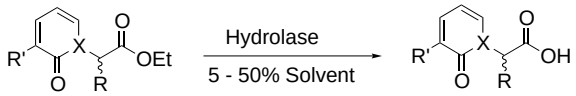
In Scheme 1 the selective hydrolysis of the *S* enantiomer of substrate **1** using *Candida antarctica* lipase B (CAL-B) as the catalyst is illustrated. In the absence of any organic solvent, the enantioselectivity (E) value of the CAL-B towards the substrate is only equal to 1.2. In order to optimize the selectivity, three rounds of solvent screening were performed. First, all solvents in Table 1 were screened at three solvent concentrations (10%, 30%, and 50%). As can be seen from the table in Scheme 1, it was found that only a select number of solvents influenced the E value of CAL-B towards this substrate, with the solvent content also playing a major role. Both dioxane and *tert*-butyl alcohol showed the most dramatic solvent effect on CALB, resulting in an enhanced E value of 3.5 at 30% solvent content, and 19 at 50% organic solvent content. These solvents were thus selected for the second round of solvent screening, where solvent content was examined in more detail. For reactions with *tert*-butyl alcohol, it was found that a solvent content of 70% gives CAL-B an E value of 25 towards this substrate. In the third round of solvent



1

Solvent	Content	E value
DMF	10	1.1
	30	1.2
	50	1.3
acetonitrile	10	1.2
	30	1.2
	50	1.2
methanol	10	1.2
	30	1.3
	50	1.2
DMSO	10	1.2
	30	1.1
	50	1.2
tert-butyl alcohol	10	1.2
	30	3.5
	50	19.1
dioxane	10	1.2
	30	3.4
	50	19.3
tert-pentyl alcohol	10	1.2
	30	2.0
	50	9.6

Scheme 1. Resolution of ester (**1**) using CAL-B. Reactions were conducted at pH 7.2, 1 mg/mL substrate, 10 mg/mL of CAL-B, 30 °C, under various solvent and solvent contents conditions. The reactions were allowed to proceed for 4 hours and were quenched and analyzed by HPLC.



R = Et, Propargyl
2

R = Et, Propargyl
3

Enzyme	[Dioxane] (%)	ee (%)	Conversion (%)	E value
Bacillus lichenformis protease	5	5	49	1.2
	35	48	45	4.1
	40	60	43	6.2
	45	66	41	7.6
	50	68	22	6.3
Aspergillus species protease	5	4	45	1.2
	35	70	12	6.2
	40	66	5.6	5.1
	45	72	2.4	6.3
	50	60	2	4.1
Bacillus lentus protease	5	30	43	2.3
	35	97	49	>200
	40	97	48	>200
	45	97	43	144
	50	98	28	143

Scheme 2. Resolution of ester (**2**). Reactions were conducted at pH 7.2, 5 mg/mL substrate, 10 mg/mL of enzyme, 30 °C, under various solvent contents of 1,4-dioxane. The reactions were allowed to proceed for 2 hours and were quenched and analyzed by HPLC.

screening, further examination using a mixture of the two best organic solvents was employed. It was found that the mixture of *t*-BuOH/dioxane/H_2O (1:2:1) slightly increased the E value to 34.

In Scheme 2, an example is illustrated where a more profound effect of solvent content, as compared to solvent choice, was observed. After initial screening of a hydrolase screening kit using 10% dioxane, no inexpensive enzyme candidates were identified that could carry out this reaction in high enantiomeric excess. A similar comprehensive solvent screen was thus initiated in attempts to enhance the E value of some potential protease candidates. All 18 solvents in Table 1 were screened, and 1,4-dioxane, amongst other potential solvent candidates, was chosen for a comprehensive solvent content screen, illustrated in Scheme 2. As can be seen from the table, all three enzymes exhibited an enhanced E value as the solvent content in the reaction mixture was increased. Moreover, *Bacillus lentus* protease exhibited a dramatic solvent content effect, having an E value of >200 at 40% solvent content as compared to an E value of only 2.3 at a 5% solvent content. Once an appropriate solvent content was identified, all 18 solvents were once again re-screened at 40% solvent content in order to identify potentially more reactive or environmentally friendly solvents for this process. As can be seen from Table 3, although the enzyme was still very selective in most solvents at 40% solvent content, the reactivity differed significantly, with some reactions not proceeding at all. Without the application of a comprehensive HTS approach, both these potential processes could not have been identified. Furthermore, this solvent effect has been found to be quite general for a variety of potential processes examined, further emphasizing the need for a routine implementation of a thorough solvent screen.

In attempts to rationalize this routinely observed solvent effect, three major arguments can be presented. First, it has been suggested that by altering solvent composition, changes in the overall enzyme structure (conformational changes) might occur giving rise to changes in the binding of the substrate to the enzyme.[16] Second, it was also suggested that the solvent can change the active site by binding in it or near it.[17] Third, it was proposed that differences in the solvation of the

Table 3. Comparison of various solvents at 40% solvent content in the resolution of ester (**2**).[a]

Solvent	% Conversion	% ee
50% glycerol	< 5	> 99
methanol	< 5	> 99
ethanol	< 5	> 99
2-propanol	29.6	99.2
acetonitrile	41.8	98.8
methyl *tert*-butyl ether	27	98.7
dichloromethane	No Reaction	–
tetrahydrofuran	No Reaction	–
1,4-dioxane	48	98.8
acetone	46	97.7
toluene	< 5	> 99
hexane	< 5	> 99

[a] Reactions were conducted at pH 7.2, 5 mg/mL substrate, 10 mg/mL of enzyme, 30 °C, under various solvent contents of 1,4-dioxane. The reactions were allowed to proceed for 2 hours and were quenched and analyzed by HPLC.

enzyme-substrate transition state complex can be a factor which influences the selectivity.[18] It is likely that this solvent effect is a combination of more than one of the above, and as such, this effect is still very difficult to predict as it is closely dependent on the structure of the substrate and the type of enzyme being screened. This reinforces the need for a comprehensive solvent screen in each case.

Conclusion

With the growing number of potential enzyme candidates that are uncovered each day as well as the dozens of solvents and solvent contents under which they can function, the search for the ideal biocatalyst for a particular process becomes more and more difficult. Despite the fact that there exists a wide range of analytical instrumentation, methods, and techniques, the choice of which methods to utilize in the screening process is essential in determining the rate of success. The implementation of the above universal screening and optimization protocols, including the one-time preparation of screening kits as well as the use of practical analytical methods has translated into not only reduced screening time and efficiency, but also significant cost savings. Furthermore, these techniques have facilitated the application of a routine comprehensive solvent screen which has led to the discovery of a number of processes exhibiting dramatic solvent effects. Although the examples discussed in this paper focus mainly on the use of hydrolases, similar screening methodologies have also been adapted for the screening of other more demanding enzymes as well as microbial libraries.

Experimental Section

Materials

The majority of enzymes utilized in the preparation of screening kits were obtained from various enzyme suppliers including Amano (Nagoya, Japan), Roche (Basel, Switzerland), Novozymes (Bagsvaerd, Denmark), Altus Biologics Inc. (Cambridge, MA), Biocatalytics (Pasadena, CA), Toyobo (Osaka, Japan), Sigma (St. Louis, MI) and Fluka (Milwaukee, WI) (See Supplementary Materials for specific enzyme sources corresponding to each enzyme). Most enzymes can be obtained from a variety of sources. Suitable 96-well plates and accessories were obtained from VWR international. Analytical instruments including the Tecan Genesis 2000 Workstation (Research Triangle Park, NC), Agilent 220 HPLC auto sampler and 6890N GC (Agilent Technologies, CA), SpectraMax Plus 384 (Molecular Devices, USA), Beckman Coulter P/ACE MDQ (Fullerton, CA), and Waters/Micromass ZQ LC/MS (Waters, MA) were purchased from their respective suppliers. Eppendorf thermomixer-R was purchased from VWR. The Lipase-PS Activity kit was purchased from Sigma. The majority of solvents utilized during optimization (Table 1) was obtained from EM Science (Gibbstown, NJ) and were of the highest purity available. Vinyl acetate and isopropenyl acetate were obtained from Aldrich. All other acylating agents were obtained from TCI America (Portland, OR). Pfizer intermediates **1** and **2** were obtained internally. Chiral HPLC columns used in analysis were obtained from Chiral Technologies (Exton, PA) and Phenomenex (Torrance, CA).

Screening and Resolution of Ester Intermediates 1 and 2

The resolution of ester intermediate **1** was carried out as follows. A level 1 screening kit was thawed for 5 minutes. 80 μL of potassium phosphate buffer (0.1 M, pH 7.2) were then dispensed into the wells *via* a multichannel pipette, and the plate was agitated for 5 minutes in an incubator/shaker. 10 μL of the substrate stock solution (10 mg/mL acetonitrile) were then added to each well, again *via* a multichannel pipette, and the 96 reactions were incubated at 30 °C and 750 rpm. The reactions were sampled after 1 hour, 4 hours, and 16 hours by the transfer of 25 μL of the reaction mixture into a new 96-well plate and were then quenched by the addition of 150 μL of acetonitrile. The 96-well plate was then centrifuged, and the organic supernatant extracted from each well into another 96-well plate. Sampled reactions were then sealed using a penetrable mat cover and transferred to an HPLC system for analysis. The same plate was used to analyze the samples for both reactivity and enantioselectivity using alternating columns on the HPLC.

CAL-B was then identified and subjected to a solvent screen as follows. 10 μL of the same substrate 1 solution (10 mg/mL) was dispensed into each well of a clean 96-well plate *via* a multichannel pipette. The solvent was then evaporated using a rotary concentrator, leaving consistent quantities of dry substrate in each well. 10, 30, and 50 μL of DMF, acetonitrile, methanol, DMSO, *tert*-butyl alcohol, dioxane, and *tert*-pentyl alcohol were then dispensed into individual wells *via* a Tecan

Genesis robotic workstation. This was followed by the addition of complementary quantities of potassium phosphate buffer (pH 7.2) required to give each well a cumulative volume of 90μL. 10 μL of a buffered solution of CAL-B (100 mg/mL, pH 7.2) were then added to initiate the reaction. The reactions were allowed to proceed for 4 hours and were then quenched and analyzed as described above. The screening for ester intermediate **2** was carried out with using the same procedures as described for substrate **1**.

Acknowledgements

The authors gratefully acknowledge Kim Albizati and Van Martin for their general support. We would also like to thank all the members from our analytical support group, mainly Jianjun Gao, Xiaobing Xiong, and Kelly Zhang for support on many of our instrument maintenance and set-up procedures. Finally, the authors would like to thank David Tat for his help in preparation of this manuscript.

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