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Applications of mass spectrometry in screening for new biocatalysts

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Abstract

Each biocatalyst screen is unique, defined by the combination of factors involved in the screen, including the number and type of biocatalysts in the screening collection, substrate chemistry and the type of assay. Advances in the technology surrounding mass spectrometry — in software, in ionization sources and interfaces and in engineering, which allows smaller mass spectrometry systems and narrow bore HPLC — have made the application of this versatile technology in screening assays possible. A mass spectrometric assay provides sensitive, specific, quantitative, high-throughput detection of new biocatalyst activities. Examples of these applications are presented and potential pitfalls are discussed.

Keywords: Biocatalysis; Mass spectrometry; Screening; High throughput; Biotransformation; Enzyme

1. Introduction

The industrial use of enzymes, or 'biocatalysts,' is increasingly popular for reasons that include the need for environmentally friendly processes, the inherent advantages of enzymes, namely selectivity and ability to operate under mild conditions, and the accumulated experience that industry has gained with biocatalysts. The three main applications of biocatalysts are (i) specific transformations, especially chiral resolutions, which are incorporated into production processes, (ii) random or directed transformations to provide analogs for lead development and (iii) transformations to produce metabolites of drugs and agrochemicals.

In order to discover new biocatalysts to meet these needs, one must embark on a search, or screen. The keys to a successful screen are sensitivity, specificity and high throughput. In this paper, we describe the application of mass spectrometry in screening for new biocatalysts, which has been made possible by advances in mass spectrometric and related technologies.

2. Incorporation of mass spectrometry into screens for new biocatalysts

Each biocatalyst screen is unique, defined by the combination of factors involved in the screen. The

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screening assay can vary widely, from a bioassay to a chemical one. Mass spectrometry is most often incorporated into the screening assay when any of the factors are applicable: (1) unpurified biotransformations are screened, with attendant complex background milieu; (2) the product lacks a suitable chromophore or fluorophore; (3) low yield/serendipitous activity is expected; (4) there is a possibility of several isomers being produced; (5) large sample numbers must be screened by a specific method; (6) quantitation is needed. Two recent reports of the use of mass spectrometry for evaluating biocatalysts illustrate the power and versatility of the technique: in the first case, for determining enantioselectivity using isotopically labeled substrates (pseudoenantiomers or pseudo-prochiral compounds) [1], and in the second case, for determining the efficiency of various amine protecting reagents in *C. antarctica* lipase catalyzed protection of amines where a uniform chromophore is lacking [2].

2.1. Recent advances in technology and their applications

The recent advances in technology that are relevant to using mass spectrometry as a screening tool are (i) more user-friendly and efficient mass spectrometry software, (ii) more versatile and easier to use ionization sources and interfaces, (iii) smaller mass spectrometry systems, and (iv) narrow bore chromatography. The software improvements make machine operation more user-friendly, allow more efficient data management and more sophisticated experiments. The electrospray (ES) ionization source was a major advancement, which allows soft ionization and sample introduction in HPLC solvents such as water, methanol, acetonitrile and some buffers. Macro- through nano-volumes are accommodated. Improvements in system engineering allow the smaller footprint needed for benchtop models. Narrow bore chromatography allows the direct introduction of column effluent into the mass spectrometer, which in turn allows quantitative experiments. These improvements translate into greater robustness, less complexity and more manageability in set-up, running and maintaining laboratory mass spectrometers.

These improvements have opened the door to new mass spectrometric applications, the most important of which in the context of this paper is the powerful application of the mass spectrometer as a versatile, sensitive, specific detector. Versatile ionization sources and interfaces allow applications for a wider variety of compounds. Interfaces allow combination with sample presentation methods ranging from narrow bore HPLC to infusion from a syringe, capillary electrophoresis/MS [3] or ultrafiltration/MS [4]. An interesting interface developed for evaluating heterogeneous inorganic catalysts [5] has potential application to biocatalysts.

Other improvements allow quantitation and provide the data handling capability necessary for high throughput screening.

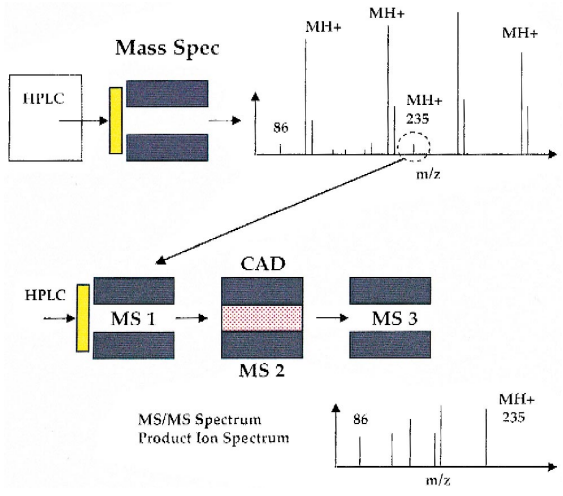

Fig. 1. HPLC-MS and HPLC-MS–MS. Top panel: In the HPLC-MS system, the eluant from the HPLC is sent into the interface region of the mass spectrometer, where the solvent is evaporated and solute molecules are ionized. Solute ions and any fragment ions with a positive charge (if operating in positive mode) enter the mass spectrometer, where they accelerate on the basis of mass, and are detected and identified upon exit. The 'full scan' mass spectrum shows several molecular ions with their sodium adducts, including a molecular ion with mass-to-charge ratio 235, and another fragment at m/z 86. Bottom panel: In the triple quadropole LC-MS–MS system, the molecular ion with m/z 235 is selected after exiting the first mass spectrometer (MS 1) for fragmentation by collision with the CAD gas in MS 2. The fragments and remaining molecular ions are accelerated through MS 3 for mass analysis. The 'full scan' MS–MS spectrum shows the molecular ion and several of its fragments, one of which has m/z 86.

2.2. The typical mass spectrometric set-up

With the improvements outlined above, HPLC-MS (Fig. 1) and GC-MS are now common, wherein a chromatographic system is used to fractionate a preparation and the mass spectrometer is positioned as a detector, monitoring the effluent. Although this paper will focus on HPLC-MS, many of the considerations also apply to screening with GC-MS. A wide variety of mass spectrometry experiments can be run. The most common are simple MS, and tandem MS (MS–MS) in which ions selected from the first MS, are in turn fragmented for further characterization in a second, linked MS. Both LC-MS and LC-MS–MS can be run in both positive and negative ion modes. In addition to the usual precursor/product scan application of MS–MS, precursor ion and neutral loss scans are possible. More directed MS–MS experiments monitor specific transitions, wherein a certain precursor ion is fragmented and monitored for the appearance of a single fragment product (Fig. 2). This mode, called Multiple Reaction Monitoring (MRM), allows detection of compounds of interest quite specifically and sensitively, while at the same time keeping data files to a manageable size. MRM is also finding application in pharmacokinetics [6,7]. Most processors and software now allow loop experiments, in which a series of different experiments are run sequentially on a single HPLC peak.

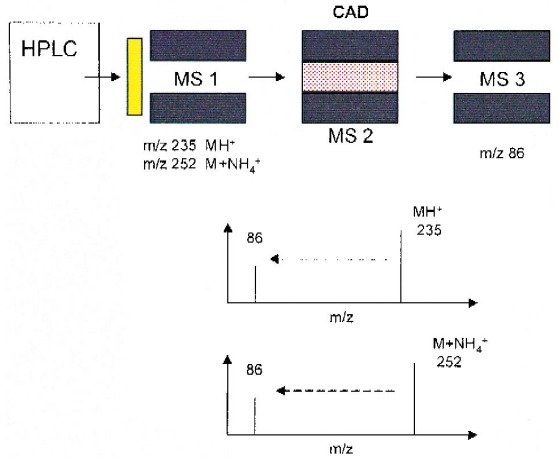

Fig. 2. MRM experiment: multiple reaction monitoring. In MRM mode, the detector monitoring ions exiting MS 3 is set to detect only those with m/z 86, a known fragment of the molecular ion with m/z 235 (and ammonium adduct at m/z 252).

2.3. Mass spectrometric strategies

Mass spectrometric strategies for screening are guided by the same parameters that guide other screens: specificity, sensitivity and throughput. For 'selective' biocatalyst screens, i.e., screens employing a smaller, focused screening population, one usually can afford to carry out more detailed analyses — longer run times per sample and full-scan MS and MS–MS. The high throughput required with a large 'random' screening population dictates faster run times per sample and more focused mass spectrometry, in which less information is gathered. Clean biotransformation extracts with one expected metabolite may be analyzed at a rate of 10 s per sample. A discussion of potential pitfalls follows, and then examples illustrate the use of mass spectrometry in a 'selective' and in a 'random' screen.

2.3.1. Potential pitfalls

As with any assay method, mass spectrometry has its areas of weakness, which often translate into lack of sensitivity. The 'soft' ionization methods such as ES and heated nebulizer are sensitive to non-volatile carriers such as DMSO, which are ionized themselves and swamp the detector when used as a carrier for direct infusion. Some normal phase chromatography solvents, notably $CHCl_3$, will dampen the ionization and result in reduced sensitivity. When the compound of interest is either fragile or does not ionize readily, again loss of sensitivity can be the result. Finally, it is important to keep the mass spectrometry experiment relatively simple — looping through several experiments by necessity reduces the sampling time for each with a resultant loss of sensitivity. In addition to sensitivity, specificity can be an issue, when there are contaminants in the substrate preparation or possible isomers of the desired product. It also is possible to design an experiment that turns out to be too specific as the actual screen unfolds: the desired product may form unexpected adducts in the reaction mixture, which are missed when running an MRM experiment. There are ways around most of these potential pitfalls; some of them are illustrated in the examples below.

2.3.2. Example 1: 'selective' screen for hydroxylation of target carbon atoms of a steroid substrate

Steroid hydroxylation has a long and successful history of commercial applications [8,9]. Despite its maturity, the area of bioconversion of steroids continues to grow, through the application of new technology to existing transformations and through new medical indications for steroid derivatives giving rise to new bioprocess needs.

For a steroid hydroxylation of a common target atom, between 40 and 100 microbes belonging to the genera listed in Table 1 are screened. Many of the strains are known to hydroxylate the target carbon atoms of a simple steroid. The remaining strains would be selected because they are versatile microbes, possibly expressing more than one hydroxylase. The important characteristics of the substrate are the degree and type of functionality introduced into the steroid molecule, i.e., factors affecting the active site fit and chemical reactivity [10]. The presence of a large number of hydroxyl groups, or bulky functional groups, tends to result in reduced metabolism of the substrate in the initial biocatalysis screen [Preisig, 1998, unpublished results].

The biotransformations are carried out in shake flasks, with starter cultures used to inoculate metabolism cultures. Two media are used for each microbe. Twenty-four hours after metabolism, cultures are inoculated and substrate is added at 0.2 to 2 μM. Bioconversions are allowed to proceed until one of two harvest times — a short time of around 6 h to minimize overmetabolism, and a longer time (24 to 48 h) for metabolically stable substrates. Upon harvest, samples are extracted with ethyl acetate, for the less polar substrates, or diluted with a miscible organic solvent, e.g., acetone, followed by centrifugation to remove insolubles and solid phase extraction.

Table 1
Genera included in a steroid hydroxylation screen for common target carbon atoms

Fungi			Bacteria
Absidia	*Diplodia*	*Phycomyces*	Bacillus
Actinomucor	*Epicoccum*	*Pycnosporium*	*Bacterium*
Amycolatopsis	*Fusarium*	*Rhizopogon*	*Comomonas*
Aspergillus	*Gibberella*	*Rhizopus*	*Mycobacterium*
Beauveria	*Gliocladium*	*Sepedonium*	*Nocardia*
Botryosphaeria	*Gongronella*	*Septomyxa*	*Pseudomonas*
Calonectria	*Hypomyces*	*Stachylidium*	*Rhodoccocus*
Chaetomium	*Lipomyces*	*Stysanus*	*Streptomyces*
Cladosporium	*Mortierella*	*Syncephalastrum*	
Colletotrichum	*Mucor*	*Thamnidium*	
Coniophora	*Myrothecium*	*Thamnostylum*	
Corynespora	*Paecilomyces*	*Thielavia*	
Cunninghamella	*Penicillium*	*Trichoderma*	
Curvularia	*Pithomyces*	*Verticillium*	
Cylindrocarpon			

The standard steroid bioconversion methodology is the primary analysis of biotransformation extracts by silica gel thin-layer chromatography (TLC) (Fig. 3) followed by secondary, reversed-phase LC-MS analysis of extracts containing metabolites (Fig. 4). UV or fluorescence activity of the products is required for sensitive detection by TLC (Fig. 3). Authentic standards are necessary to confirm the structural characterization of compounds using LC-MS and MS–MS techniques.

A simple, yet powerful, method for distinguishing isomers from the desired product is illustrated in Fig. 4 for isomers of monohydroxy-4-androstene-3,17-dione (OHAD). While 11α-OHAD and 11β-OHAD are well resolved by reversed-phase HPLC, the regioisomers, 9α- and 11α-OHAD, and other steroids are not well enough resolved to be distinguished in a biotransformation screen. However, between pairs of 9α- and 11α-isomers, the 9α-isomers fragment 10 times more readily. Many steroids fragment very little in ES mass spectrometry, producing only the molecular ion $[M + H]^+$ and the ammonium adduct. The presence of a hydroxyl group is observable, in the $[M - H_2O]^+$ fragment ion. In Fig. 4, the 9α- and 11α-OHAD ($[M + H]^+ = m/z$ 303) can be distinguished with these ions: the 9α-OHAD forms the ammonium adduct (m/z 320) and readily fragments, losing H_2O (m/z 285, 267 and 249), whereas 11α-OHAD does not. The difference in the ratio of intensities of the molecular ion to any of the three most abundant fragments is sufficiently consistent to be diagnostic and, thus, the basis for a screen. The limit of detection for steroids is in the tens to hundreds of nanograms (1 nM) range using ES mass spectrometry.

If the substrate does not have suitable chromophore or fluorophore, and/or standards are not available for the desired products, then the TLC to

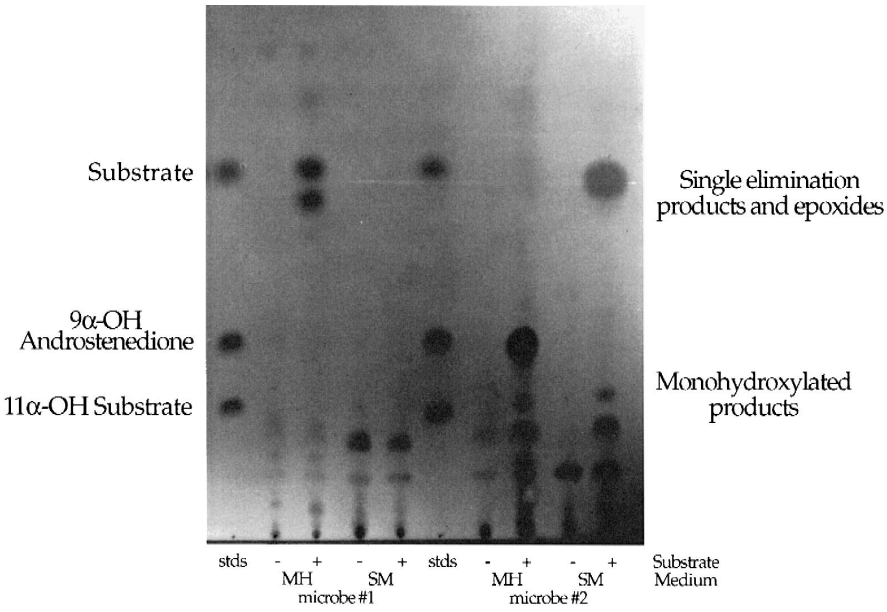

Fig. 3. Silica gel TLC of ethyl acetate extracts from 24-h biotransformations screened for hydroxylation of a target carbon on a steroid. Biotransformation extracts, with and without substrate, for two microbes in two media (MH and SM) were spotted on silica gel 60 plates with fluorescence backing. Lanes, left to right: (1) standards (substrate, 9α-OHAD, and 11α-hydroxylated substrate), lanes (2) to (5) four microbe #1 extracts, (6) standards, lanes (7) to (10) four microbe #2 extracts. The substrate concentration was 0.1 g/l; 2% of the ethyl acetate extract was spotted per lane in 10 μl. Plates were developed in $CHCl_3$:CH_3OH, 95:5, v:v, and visualized with a short wave UV lamp.

LCMS sequence is less useful. Instead, the primary analysis can be LC-MS, followed by LC-MS–MS of extracts containing possible hydroxylation products. Without authentic standards, the retention time of desired products is estimated, with a related, more polar steroid to estimate the outer range of retention times to expect. Anything eluting between this compound and the substrate is considered a potential product. If standards are not available, the second, MS–MS step is critical to confirm fragmentation patterns.

Fig. 5 presents a biotransformation in which more than one hydroxylated product was detected based on HPLC retention time. Using MS, the most abundant product, which was not resolved from the substrate, was detected. This bioconversion was run in four media — with a different yield of this product in each medium. Yield was 10% (without optimization) — a low yield for most steroid bioconversions — but due in part to the fact that this particular substrate was highly functionalized to begin with. In this study, the microbes with a history of activity on the target carbon atom were not the most active in the screen — which might have been expected because of the highly functionalized nature of the substrate. In addition to the fit of the substrate in the active site, other factors that affect yield and predictability in hydroxylation screens using microbes are substrate solubility, its ability to be absorbed by the microbe, and its effect on other aspects of microbial metabolism, including induction of hydroxylases.

2.3.3. Example 2: large microbial screen for rare / serendipitous enzyme activity

The biocatalyst screen in this example followed on an earlier, unsuccessful screen of 4000 microbes by a less sensitive method [unpublished results]. The desired enzymatic activity was rare — however, even very low activity, which could be used as a starting point for directed protein evolution, was of interest. For the screen, 25,000 microbial extracts

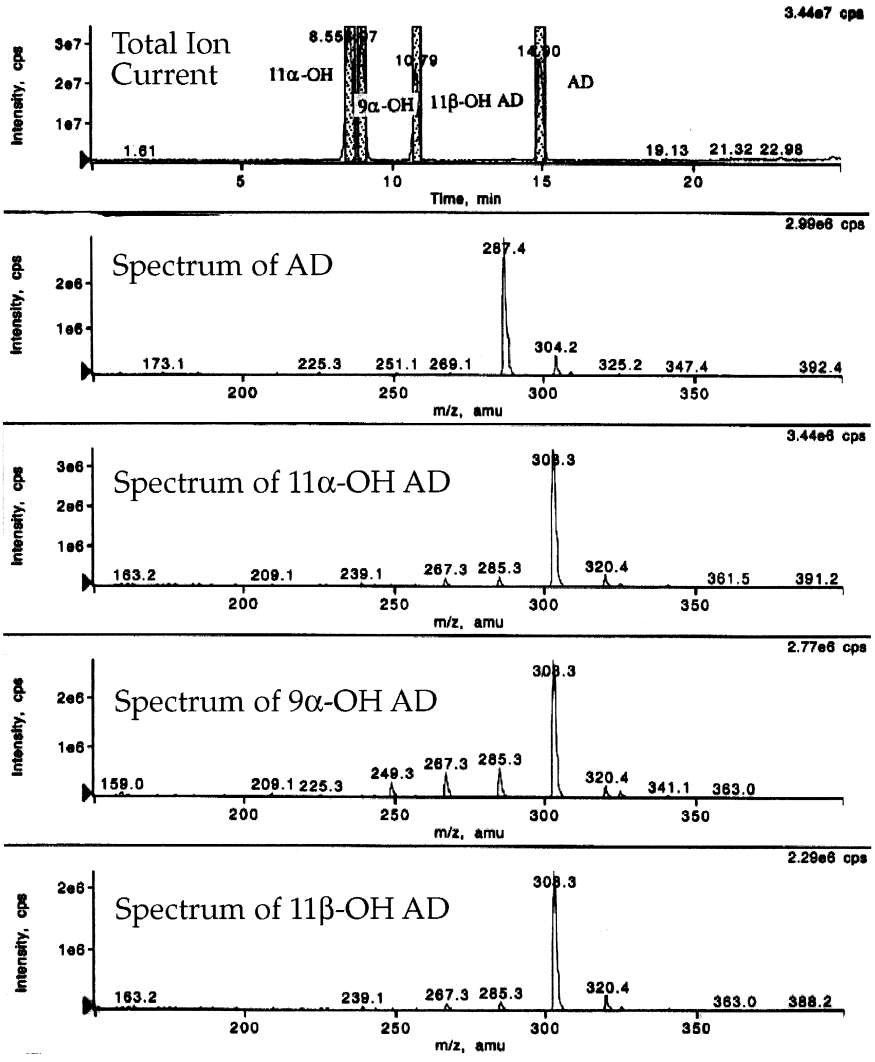

Fig. 4. LCMS of androstenedione and three monohydroxylated derivatives. Reversed phase HPLC on Nova-Pak C-18 with a 25-min linear gradient from 75:25 to 25:75 H_2O:CH_3CN. Top panel: Total ion current. Retention times are 8.55, 8.97, 10.79 and 14.90 min for 11α-OHAD, 9α-OHAD, 11β-OHAD and AD, respectively. Bottom four panels: Mass spectra of the individual peaks, showing molecular ions (AD, m/z 287, and OHAD, m/z 303) ammonium adducts at $(M + H) + 17$ m/z, and fragments due to loss of H_2O at $m/z = (M + H) - 18, -36, -54$.

were analyzed for the biotransformation of the substrate to the desired product, which did not contain a useful chromophore or fluorophore. The microbes were tested in shake flasks in one or two media. Media recipes were varied; a total of 16 different media were used for the initial screen.

Given the lack of a suitable chromophore, and the low level of bioconversion expected, mass spectrometry was the analytical method of choice. Ethyl acetate extracts of the biotransformations were complex enough that an LC-MS–MS MRM experiment was required, rather than a LC-MS or MRM without chromatography, in part because the substrate preparation used for the biotransformations contained significant amounts of contaminants which were closely related to the substrate. The limit of detection of the

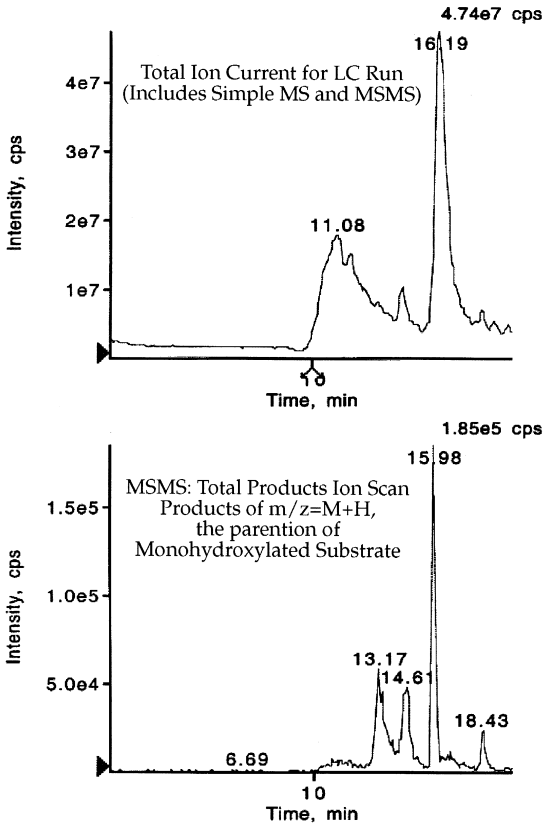

Fig. 5. LC-MS and LC-MS–MS of the biotransformation extract from a screen for the monohydroxylation of a highly functionalized steroid. An equal volume of methanol was added to the biotransformation and the mixture was centrifuged. The supernatant was reduced in volume in a speed vac, and loaded onto an Oasis cartridge. After washing with water, products were eluted with 80% aqueous methanol and dried in a speed vac. The dried extract was solubilized in methanol for HPLC-MS–MS analysis. Top panel: Total ion current for the LC run (reversed phase) in a loop experiment combining MS and full scan MSMS. The retention time for the substrate is 16.19 min. Bottom panel: LC-MS–MS current only, for products of the molecular ion for the monohydroxylated substrate. Monohydroxylated products were confirmed for the peaks at 13.17, 14.61 and 15.98 min.

LC-MRM method was about 10 pg on column. With initial substrate concentrations of 0.5 to 1.0 g/l, very low conversions were detectable. The LC method was isocratic; the analysis time was 5 min per sample. The system, a Gilson 233XL fraction collection, Waters HPLC and PE-Sciex API3 + mass spectrometer equipped with TurboIonspray, was robust. This permitted runs of 200 samples plus continuing standards. The system was run reliably, with twice weekly overnight recycles, for the analysis of over 27,000 samples.

The information obtained by LC-MS–MS — retention time and MRM pattern — allowed candidate identification (Table 2). The candidates needed to be analyzed further to confirm (or deny) the product, since the substrate was a complex molecule for which several product isomers were possible. Approximately 20 candidate microbes were identified during the initial screen out of 15,900 screened. The highest level of conversion was about 0.001%. The candidate peaks were purified from several larger scale runs and confirmed by full scale MS–MS. Follow-up for such low-level conversions needs to be very sensitive, LC-NMR. Even so, at 0.001% conversion, this requires scale-up to 5 l to obtain enough products for LC-NMR.

2.3.4. Example 3: hydroxylation of a macrolide — development of a microbial transformation process

The goal of this screen was to find a biocatalyst for the stereoselective hydroxylation of a macrolide. The substrate macrolide itself was expensive, so in addition to high product yield, it was important to find a biocatalyst that efficiently utilized the substrate for the bioprocess. The biggest problem ini-

Table 2
Transitions for the MRM experiment for high throughput screen

1st Quadrupole	3rd Quadrupole
$[M+H]^+$ Molecular ion	$M-H_2O$[a]
	$M-[H_2O+\text{func grpA}]$
$[M+NH_4]^+$ Ammonium adduct[b]	$M-H_2O$[c]
	$M-[H_2O+\text{func grpA}]$
	$M-[2H_2O+\text{func grpA}]$
	$M-\text{func grpB}$

[a] Unusable due to frequent high background from the media and microbes. With the high throughput required in an initial screen, 'no substrate' controls cannot be run.

[b] Ammonium adduct was 10 times as abundant as molecular ion. Sodium adduct, which also was present, does not fragment.

[c] Unusable due to the presence of a similar transition from a contaminant in the substrate preparation that was present at 1% to 2%.

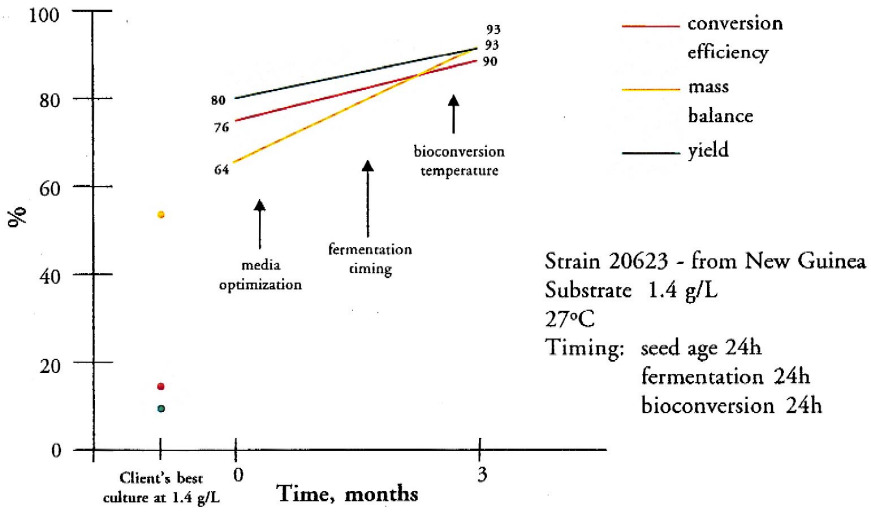

Fig. 6. Optimization of the strain 20623 bioprocess for the hydroxylation of a macrolide. The performance of the best strain at 1.4 g/l at the outset of the screen (single points) is compared with the performance of the best strain from the initial screen (at time = 0 months). The progress of process optimization, primarily focused on medium optimization, fermentation timing and bioconversion temperature, is indicated by the three colored lines. After 3 months of work, performance for all three parameters reached greater than 90% at 1.4 g/l in a 24-h bioconversion at 27°C.

tially appeared to be substrate intolerance by the starting strain. When the substrate concentration was raised to 1.4 g/l, the yield dropped to 8%, with a conversion efficiency of 12% and mass balance of 55% (Fig. 6). The goals of this screen were to find a microbe, which converted the substrate to product with yield, mass balance and conversion efficiency all greater than 70% at 1.4 g/l of substrate. This example will focus on the process optimization work that followed the initial screen.

For this project, 1500 new microbes were isolated from a specific habitat type and 10,000 microbes were screened in total. The microbial transformations were carried out in shake flasks to ensure adequate microbe growth and adequate aeration during the bioconversion. One medium was tested per microbe during the initial screen; media recipes were varied for a total of seven media in the screen.

Four strong microbes were discovered which met the client's criteria of 70% yield, conversion and mass balance. As with any process, the initial results usually can be improved upon. Several aspects of the bioconversion were studied with these four strains, namely, seed process, medium optimization, fermentation time course, the effect of feeding glucose and substrate tolerance. With 3 months of process development work, the performance of strain 20623, from New Guinea, was greater than 90% for yield, mass balance and conversion efficiency at 1.4 g/l.

3. Conclusions

Mass spectrometry is useful for high throughput screening for new biocatalysts when any of the following are involved: (1) microbial transformations are screened, with attendant complex background milieu; (2) the product lacks a suitable chromophore or fluorophore; (3) low yield/serendipitous activity is expected; (4) there is a possibility of several isomers being produced; (5) large sample numbers must be screened by a specific method; or (6) quantitation is needed. The particular aspects of each screen will differ; requiring appropriate experimental design to detect the desired activity and avoid pitfalls. With an appropriately designed system, screening for low level bioconversions in complex matrices by mass spectrometry can readily handle more than 200 samples per day; clean samples can be analyzed in 10 s apiece.

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