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Asymmetric bioreductions: application to the synthesis of pharmaceuticals

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Abstract

Selected examples of asymmetric bioreductions of pharmaceutically relevant prochiral ketones are reviewed. These data show that microbial screens lead to the identification of appropriate biocatalysts, and that the use of miniaturized and semi-automated technology can greatly reduce both labor and lead times. The same data also highlight the need to evaluate a relatively large and/or diverse microbial population (highlighting biodiversity). We also found that in many instances the luxury of producing either enantiomers with high optical purity, enantiocomplementarity, can be achieved when employing different microbial strains. Process development studies reviewed here demonstrate that it is possible in some cases to understand and control the production of an unwanted enantiomer or by-product. Finally, a specific example, the asymmetric bioreduction of a ketone by *Candida sorbophila*, shows that process development studies which optimized, the bioreduction environmental conditions (pH, temperature...), the addition of ketone, and the implementation of a nutrient feeding strategy in conjunction with the use of a defined cultivation medium were key in achieving increased bioreduction rates and product titers. When scaled-up in pilot plant bioreactors, the bioreduction process supported the production of several kilograms of (*R*)-alcohol (enantiomeric excess (e.e.) > 98%), with an isolated product yield of about 80%.

Keywords: Assymetric bioreductions; Ketones; Process development, fermentation

1. Introduction

Recent investigations have clearly established that enantiomers of racemic pharmaceutical drug mixtures can present different pharmacokinetics and bioavailability properties. The manufacture of the active form of the drug is consequently becoming a norm in the industry [1–3]. Despite tremendous scientific and technical advances, however, several chiral syntheses still remain difficult and/or expensive and asymmetric biocatalysis employing either whole microorganisms or isolated enzymes has often emerged as a viable alternative [4–7].

Chiral alcohols are very important precursors for a large number of pharmaceuticals. Their production by asymmetric bioreduction of a prochiral carbonyl precursor is becoming well-established in the field of biocatalysis [8–10]. This communication summarizes some of our achievements in this field, focusing on

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the microbial screening, process development, and scale-up activities.

2. Approach to the implementation of bioconversions

Our approach to introducing a biotransformation step in the synthesis of small pharmaceutical molecules is initiated by the identification of a specific synthetic step where a bioconversion could be of interest. Once it is established that a biocatalyst would present technical and/or financial benefits, decisions of technical feasibility are then made based both on internal expertise and by consulting bioconversion data bases [11,12]. Procedures for screening microorganisms or enzymes that catalyze the desired step are then designed and implemented. After successful completion of the screening, a process is developed at laboratory scale and later scaled-up in the pilot plant. These basic approaches are outlined in Fig. 1.

3. Microbial library construction

Generally, the efficiency of all microbial screens are based on the construction and accessibility of a microbial library. Ideally, this library should be comprised both of pre-selected microbial strains with proven or potential desired activity and should be large enough to ensure a likely positive and timely outcome to the screening effort. Our approach to constructing a suitable microbial library for the asymmetric bioreduction of ketone intermediates evolved over time as internal expertise and a data base were gradually built. We initially capitalized on both published literature, which described microbial strains with bioreducing activity (available through external microbial culture collections), and on the privileged access to several very large and ecologically diverse microbial culture collections. Based on prior bioreduction art [4–9], we elected to focus our screen on yeast strains and, to a lesser extent, fungi and bacteria. Most of the yeasts evaluated were out-sourced from the culture collection at the University of California, Davis, which indubitably represents a collection of diverse species from a wide range of ecological origins. Most fungi and bacteria were obtained from the Merck Microbial Culture Collection, which also guaranteed us access to a very

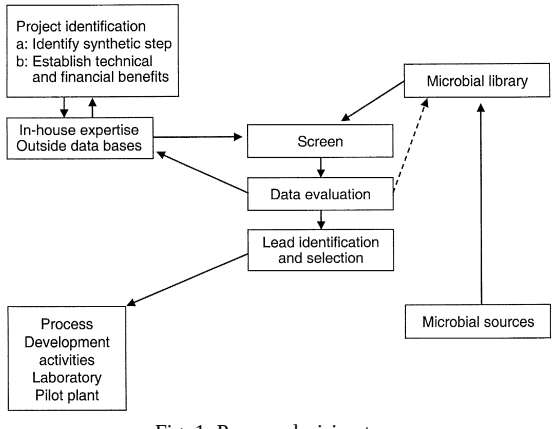

Fig. 1. Process decision tree.

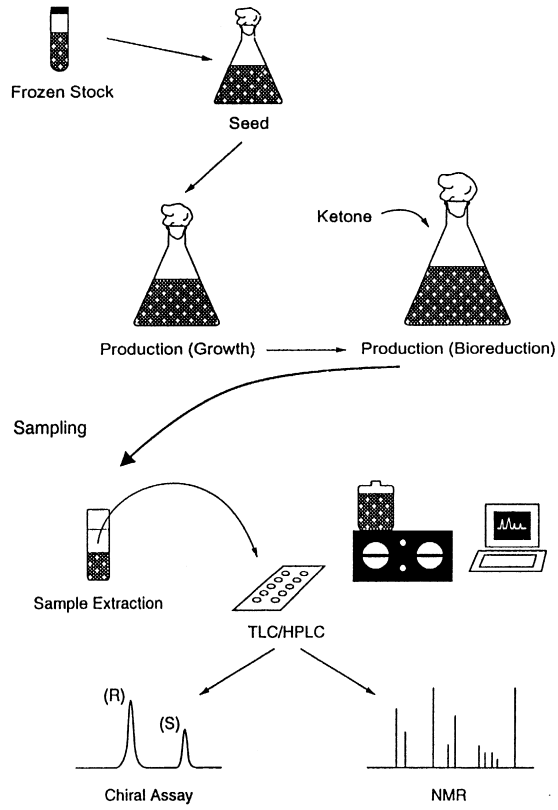

Fig. 2. Classical screen for bioconversion activity.

large and ecologically diverse source of microbes. Additional microbes were isolated directly from environmental samples and added to our bioreduction microbial library.

4. Screening methodology

The screening method used for detecting asymmetric bioreduction activity is summarized in Fig. 2. Until the development of a semi-automated screening method, which is described later, most screens relied on the aerobic cultivation of the microbes in test tubes or small Erlenmeyer flasks (250 ml) containing an appropriate cultivation medium. While the ketone substrate can usually be added upon inoculation, we found that substrate additions made to late growth phase cultures were preferable. These delayed additions may also help to circumvent potential ketone toxicity to the microbes in their early growth phase. Most ketones that were used in our screens exhibited low aqueous solubility, and the addition of a biocompatible solvent such as ethanol or DMSO (approximately 2%), was routinely employed. The use of these solvents achieved both the ease of dispensing a solution rather than a solid to the culture flasks and increased the solubility of the ketone substrate in the reaction medium. After incubation in the presence of the ketone, which typically ranged from 24 to 96 h, the cultivation broth was sampled and tested for the presence of the desired alcohol by either thin layer chromatography or preferably by high pressure liquid chromatography (HPLC). The enantiomeric excess (e.e.) of the produced alcohol was routinely evaluated by HPLC, employing specific chiral columns. The most promising strains were usually re-cultivated in 2-l flasks, and after proper isolation, the identity of the alcohol produced was verified by nuclear magnetic resonance analyses.

Once our bioreduction microbial library was established, the need to increase the efficiency of our screening operations (by reducing time and labor) became obvious. To address this issue, we developed a miniaturized and automated method that allowed us to evaluate several hundred microbial strains in a relatively short period of time [13]. This method uses

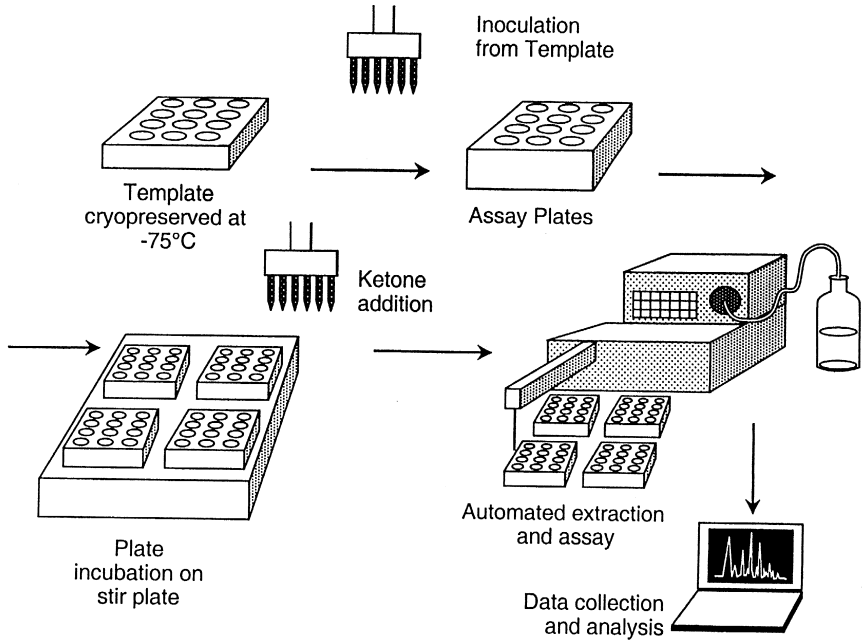

Fig. 3. Miniaturized and semi-automated screening method.

24 well plates, each 10-ml well containing 2 ml of cultivation medium and a small stir bar. Each plate is inoculated from a similar (plate) cryopreserved at −7°C, containing a different microbial strain in each well. After an incubation period of usually 24 h with stirring, the ketone substrate is added in a biocompatible solvent, and the plates returned to the incubator. The stirring provides aeration and adequate mixing, especially when using non-water soluble ketones. Sample preparation and assay are then performed by a liquid handler/HPLC system which requires minimal supervision. Fig. 3 present a diagram of the screening operations associated with our faster and more efficient method. Based on the capacity of our miniaturized and semi-automated screening system and on the fact that most screens require the evaluation of less than 250 strains (data presented below), the identification of most reduction biocatalysts should take no more than a few days.

5. Microbial library properties

Fig. 4 summarizes the diverse asymmetric bioreductions that were performed over the past few years at Merck on a large variety of ketone substrates. It shows that the range of substrates acceptable for bioreduction was quite large and diverse. It includes cyclic ketones and diketones (**1**,**7**), several very sterically hindered structures (**3**,**8**), substrates with an hetero-atom in the vicinity of the carbonyl bond of interest (**5**), as well as some poorly water soluble substrates (**1**,**5**).

The data presented in Table 1 show that for some applications, it was necessary to only screen a very limited number of microbial strains in order to identify a suitable biocatalyst. In most cases, the evaluation of less than 150 strains provided us with one or several acceptable biocatalysts. A remarkable hit rate of 100% was observed for compounds (**5**,**9**,**11**) while

Table 1

Compound	Strains tested	Hit rate (%)	Enantiocomplementarity	Microorganisms yielding the desired enantiomer with highest e.e.
1	80	50	yes	*T. capitatum* MY 1890
2	35	37	no	*C. schatavii* MY 1831
3	129	< 1	no	*C. magnoliae* MY 1785
4	260	< 1	no	*M. alpina* MF 5534
5	14	100	yes	*C. sorbophila* MY 1833 *Kluyveromyces delphensis* MY 1476
7	32	53	yes	*T. cutaneum* MY 1506 *C. Kefir* MY 1717 *S. johnsonnii* MY 1673
8	310	3	yes	*R. pilimanae* ATCC 32762 *Aspergilus nidulans* MF 121 *Bullera tsugae* MY 1669 *Mucor sulfa* MF 37
9	9	100	yes	*R. rubra* MY 2169 *R. pilimanae* ATCC 32762
10	144	10	yes	*C. magnoliae* MY 1785 *Arxiozyma telluris* MY 1927 *Zygosaccharomyces montanus* MY 1919
11	20	100	yes	*Hansenula subpelliculosa* MY 1552 *Pichia delftensis* MY 1569 *Kluveromyces marxianus* MY 1516 *S. bayanus* MY 1930 *R. pilimanae* ATCC 32762 *P. carsoni* MY 1622
12	51	24	yes	*R. pilimanae* ATCC 32762 *H. minuta* MY 1546

a more extensive screening effort had to be undertaken for a few ketones (**3**,**4** and **8**). While no definite explanations are yet available for low hit rates, we speculate that poor ketone transport through the membrane, and/or low enzyme affinity for a specific ketone may be two of several possible explanations. Data presented in Table 1 also show that for most ketones evaluated (**1**,**5**,**7**–**12**), enantiocomplementarity, the production of both enantiomers, could be achieved when using a different strain. This feature confers an added versatility and attractiveness to microbial asymmetric bioreductions. Results summarized in Table 1 also show that in some cases, the screen results provided us with a choice of several biocatalysts. These data also highlight that for each application, a different microbial strain was selected as the most desirable catalyst to use for further process development activities. Generally, strains that performed adequately in a specific screen did not necessarily perform well during prior or subsequent screens. A few noticeable exceptions, *Rhodotorulla piliminae* (ATCC 32262), *Candida magnoliae* (MY 1785), *Trichosporon capitatum* (MY 1830), *Yarrowia lipolytica* (ATCC 48436) *C. sorbophila* (MY 1833) and *Sporidiolobus johsonii* (MY 1673), presented bioreducing activity in several screens (Table 2). However, only *R. piliminae* (ATCC 32262) and *C. magnoliae* (MY 1785) are the only two strains that performed remarkably well in several screens (Table 2). Interestingly, *Saccharomyces cerevisiae* (baker's yeast) which is very often mentioned in the literature as the catalyst of choice for asymmetric bioreductions [4,5,7,9] was never selected as a desirable biocatalyst for any of the substrates that we evaluated.

Table 2

Microbial strain	Substrate (bold numbers from Table 1) and e.e. of the desired alcohol (%)
R. pilimanae ATCC 32762	(**8**; 96) (**9**; 100) (**11**; 95) (**12**; 99)
C. magnoliae MY 1785	(**3**; nd[a]) (**7**; 99) (**10**; 97)
T. capitatum MY 1890	(**1**; 71/100[b]) (**2**; 72) (**12**; 43)
Y. lipolytica ATCC 48436	(**1**, 0) (**12**, 83)
C. sorbophila MY 1833	(**5**, 96) (**12**, 49)
S. johnsonii MY 1673	(**2**, 92) (**7**, 99) (**10**, 60)

[a] Production was too low to determine e.e.

[b] The e.e. was increased from 71% to 100% as a result of process development.

6. Process development and scale-up

The selection of a strain to be used for further process development work is performed based first on its enantioselectivity and second on the apparent conversion yield observed during preliminary studies.

Bioreductions can be either performed by using isolated enzyme or by employing whole cells. Both approaches present advantages and inconveniences. Due to the presence of many enzymatic activities, whole cell processes may yield the production of by-products. Also, the final product has to be purified from a cultivation medium containing cells and spent cultivation broth. To the contrary, isolated enzyme processes tend to simplify product isolation and are unlikely to form undesirable by-products. However, while isolated enzyme processes appear to be very attractive, their use in bioreduction processes do present some inconveniences and major technical challenges. First, the production, isolation, and stabilization of the desired enzyme need to be developed and scaled-up. Second, the enzymatic reduction of carbonyls to alcohols requires the transfer of hydrogen and electrons usually from the co-factor NADH,H (or NADPH,H). An enzymatic bioreduction process will therefore either require the stoichiometric addition of the very expensive co-factor or the in vitro regeneration of the co-factor. The in vitro implementation of co-factor recycling on an industrial scale has not yet been demonstrated to be economical and practical. On the other hand, whole cells offer the advantage of efficient and economical in-vivo co-factor regeneration. Additionally, microbial process development and scale-up offer the advantage of drawing on several decades of industrial expertise. For these very reasons, we currently implement whole cell bioreduction processes at the large scale.

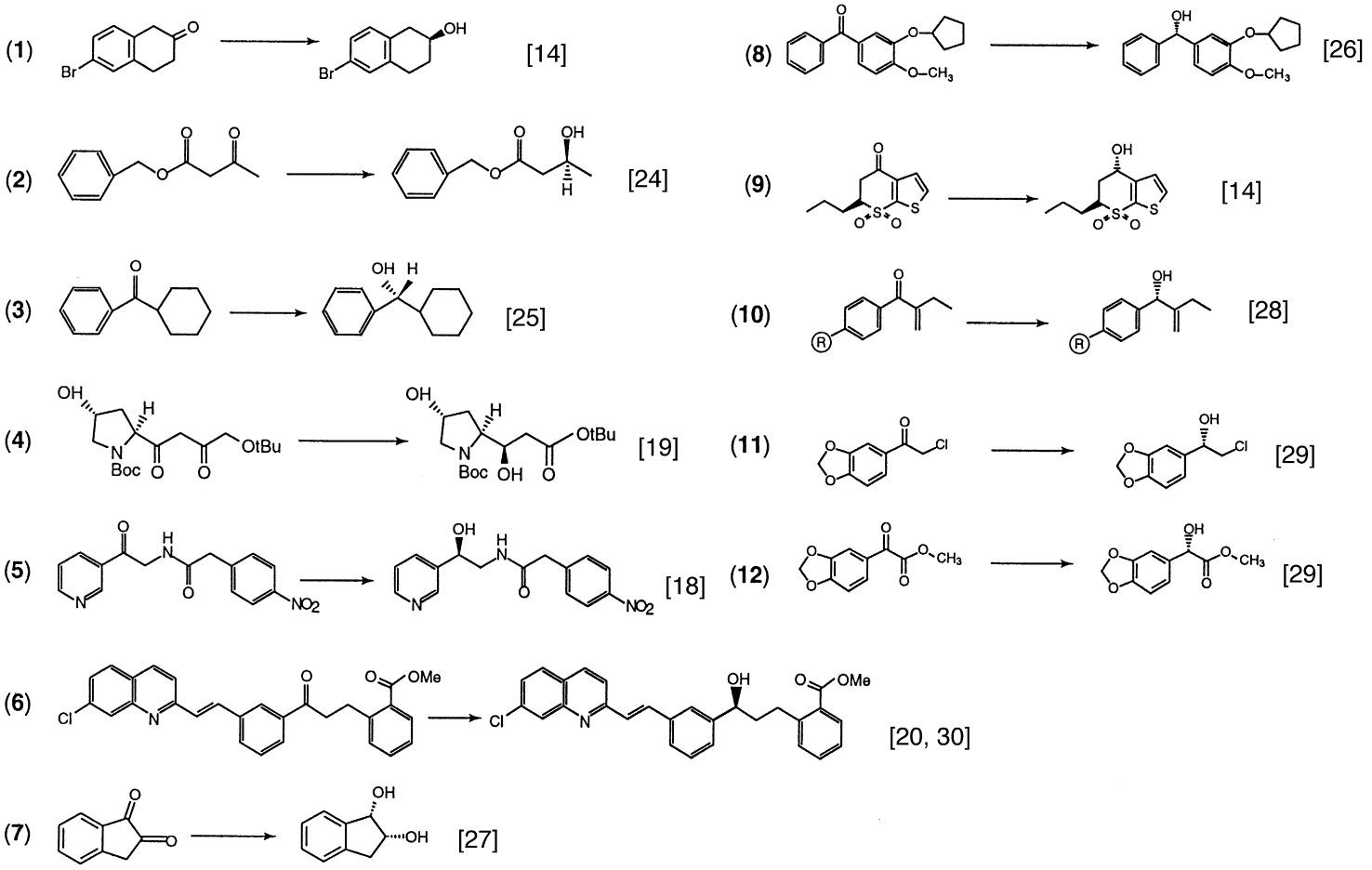

Fig. 4. Bioconversion of pharmaceutically relevant ketones. The numbers in brackets are the references to the published literature [14,18–20,24–30].

7. Bioreduction process issues

The most important issues when developing a bioreduction process are (in order of importance) achieving high e.e., high conversion yield and high product titer.

Because we elected to employ whole cells, which are inherently metabolically complex, a potential exists for the formation of by-products and/or unwanted enantiomer. It is therefore imperative that these phenomena be understood and controlled. Sometimes, the control of unwanted by-products is simple. For example, the formation of by-products in the bioreduction of beta-bromotetralone was observed when the temperature of the reaction was above 32°C [14]. By carefully controlling bioreduction environmental conditions, the formation of these unwanted by-products were routinely averted. More challenging is when the microbe selected for the scale-up continues to produce undesirable amounts of the unwanted enantiomer. This undesirable synthesis is usually attributed to the presence of a second oxydoreductase enzyme with opposite selectivity with respect to the desired activity [15,16]. In one particular study, we discovered that the unwanted activity could be controlled by careful selection of the cultivation conditions, namely by cultivating the microbes in the absence of glucose [14]. We speculate that the second unwanted enzyme induction or activity is tightly regulated by glucose or one of its metabolites. By implementing a cultivation medium devoid of glucose, the scaled-up process produced the desired (*S*)-alcohol with an e.e. greater than 99%, a remarkable improvement over the initial values which were hovering around 70%. In another study, we found that the unwanted enzyme activity, responsible for the formation of the undesired diastereoisomer, had a widely different affinity for the ketone substrate [17]. Production of the unwanted *trans*-diastereoisomer usually occurred when the external concentration of the ketone substrate fell below a specific value (less than 400 mg/l). By controlling the ketone substrate residual concentration and by carefully timing the harvest, we were able to control the unwanted enzyme activity and to produce a *trans*-hydroxysulfone with an acceptable diastereomeric excess. Due to this improvement, the optical purity of the desired *trans*-diastereoisomer was raised from a low value of about 90% to 96%, allowing this material which now met purity requirements to be used in the subsequent steps of the synthesis of the final compound [17]. Both of these examples demonstrate that once we understood the metabolic events controlling the undesirable activity, production of alcohols with an acceptable optical purity could be routinely achieved. In many other cases, we were fortunate enough to identify a microbe with probably only one oxydoreductase which presented affinity for our specific substrate, and therefore achieved elevated alcohol e.e. irrespective of the environmental cultivation and bioconversion conditions [19–20].

As mentioned earlier, after achieving elevated e.e., obtaining high yield and product titers are the next two most important factors to consider. These two factors will greatly influence both process scalability and economics. In order to achieve these goals, we routinely evaluate the influence of cultivation medium composition, microbial growth and bioreduction environmental parameters (pH, temperature, etc.), and the effect of nutrient and ketone feedings on process performance [14,19–20]. We usually approach parameter-optimization studies using efficient computer-based statistical experimental designs [21]. For example, the bioreduction of ketone (**4**) to its (*R*)-alcohol by the yeast *C. sorbophila* (Fig. 4) illustrates perfectly how an efficient bio-process can be developed and scaled-up using statistical designs [18]. Chemical chiral methods have so far only produced materials with unacceptable e.e. [18]. In order to facilitate process scalability, in-process monitoring and downstream product purification, we developed a chemically defined cultivation medium. These studies were followed with the optimization of both

Table 3

Bioreduction process characteristics	
Chemically defined medium (glucose, yeast nitrogen base, monosodium glutamate, copper chloride)	
Growth phase (0–20 h)	uncontrolled pH from initial of 6.5, 34°C aerobic
Bioconversion phase (20–120 h)	pH controlled at 8.0 glucose feeding (1.5 g/l/h) 34°C aerobic (DO > 30%) ketone addition in 0.9 M sulfuric acid

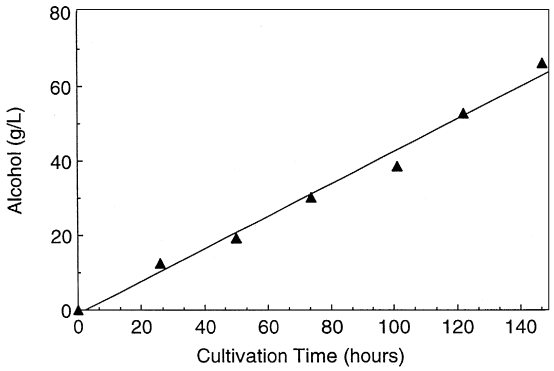

Fig. 5. Bioreduction potential. A culture of *C. sorbophila* was used to bioconvert 60 g/l of substrate (**5**) to its corresponding (*R*)-alcohol.

the environmental conditions and feeding strategies employed during both microbial growth and bioreduction phases of the process. Table 3 lists the optimized process conditions that were used in subsequent scale-up studies. This well-tuned process was found capable to bioreducing up to 60 g/l of ketone to its (*R*) alcohol with analytical yields close to 100% (Fig. 5). Due to a lack of ketone availability, no higher ketone concentration could be evaluated at the time. Prior to scale-up, a key challenge was to sterilize the ketone. When processes are used for the production of clinical materials, all operations must comply with current Good Manufacturing Procedures (cGMP) where the requirement for a well-controlled process dictates that all solutions fed to a microbial cultivation vessel be aseptic. Heat treatment and organic solvents were excluded for deleterious effects and lack of sufficient solubility, respectively. Only elevated ketone solubilization was achieved when using sulfuric acid (0.9 M) and then was filter-sterilized employing appropriate filters. A full process pictorial presented in Fig. 6 shows that we heavily relied on both on-line and off-line monitoring. Fig. 6 also shows that the routine use of on-line double filters ensured sterile operations. Fig. 7 presents a typical run performed with an initial ketone charge of 20 g/l. Due to the low aqueous solubility of the ketone (< 0.5 g/l) and of the

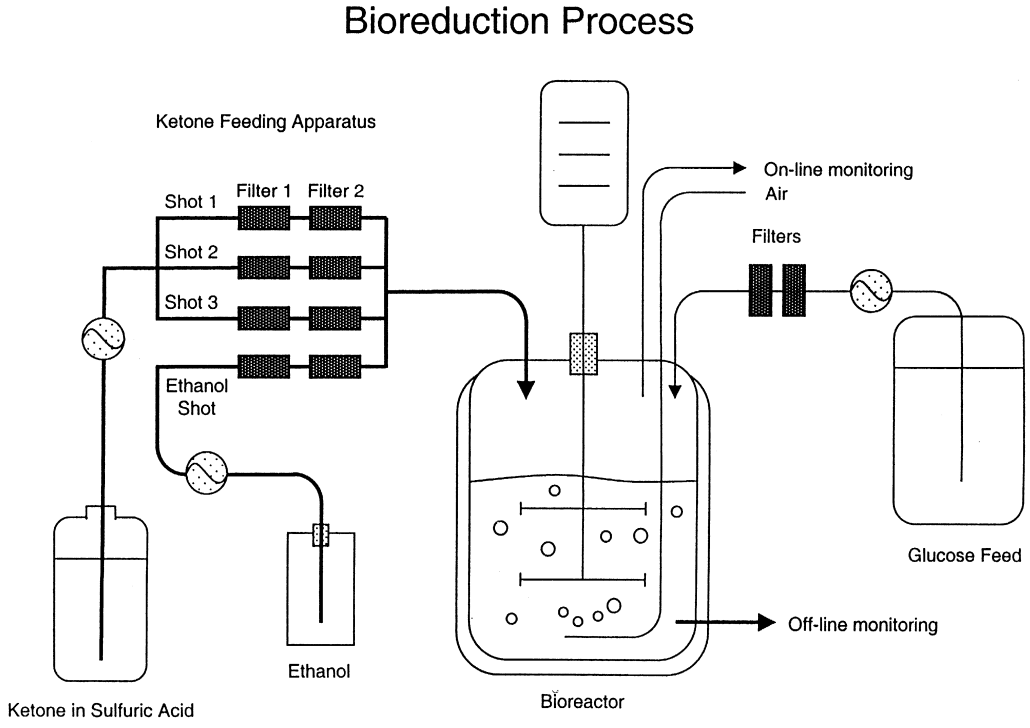

Fig. 6. Bioreduction process pictorial. *C. sorbophila* was cultivated in a 260-l bioreactor, employing the conditions described in Table 3.

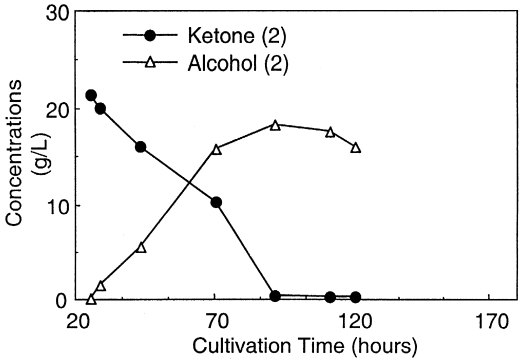

Fig. 7. Bioreduction kinetics. The asymmetric bioreduction of ketone (**5**) to its corresponding (*R*)-alcohol was performed as described in Table 3 and in this figure.

alcohol (about 2.5 g/l), mass balance calculations on in-process samples were difficult and thus explain the erratic bioconversion kinetics presented here. In the end, and after several purification steps which include cell removal and solvent extraction [18], this process successfully produced several kilograms of clinical material, achieving very reasonable yields (80% on isolated product) and excellent alcohol e.e. (> 98%).

8. Conclusions and future directions

The studies summarized here clearly show that asymmetric bioreductions can be a very valuable alternative to chemically difficult chiral reductions. More specifically, biocatalyst identification for asymmetric bioreductions has proven to be a quite reliable and timely exercise in many examples. The microbial library that we assembled has performed remarkably well during all the screens described here as reflected by the identification of appropriate biocatalysts. Most remarkable is that in many of these examples, less than 250 microbial strains had to be evaluated in order to yield an appropriate biocatalyst candidate. Our data also show that asymmetric bioreductions offer the advantage of accepting a large array of pharmaceutically relevant ketone substrates, and in some instances offer the luxury to produce, when employing a different microbial strain, each enantiomer with high e.e. We realize however that shortening lead time for biocatalysis discovery must be taken into account, and certainly the use of our miniaturized and semi-automated microtiter plate-based microbial library should positively contribute toward that goal.

Process development studies have demonstrated that optical purity can, in some cases, be controlled and, that yields and alcohol titers can be optimized to yield viable processes. More importantly, these processes can be scaled-up and support the efficient production of large amounts of high quality materials.

We believe that there are several paths toward improving this valuable technology. Screening for the appropriate biocatalyst can be further automated and improved, especially by the addition of relevant strains to the microbial library, so that it delivers, in most cases, an excellent biocatalyst in an acceptable period of time. The isolated enzyme approach should certainly be extensively studied. The production of the desired enzyme, more likely via homologous or heterologous over-expression should be optimized and coupled with an appropriate formulation and stabilization. Enzyme reactor technology development and co-factor recycling are the logical steps that must follow. The now well-established enzyme-directed evolution technology [22] should also be used to adapt the desired enzyme to the relevant process constraints. Finally, when the subsequent step to bioreduction can also be performed by biocatalysis, one could also envision to perform both bio-steps in the same microbial host, employing the tools of metabolic engineering [23].

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