

Using Biocatalysts at Useful Reactant and Product Concentrations

Monitoring and Controlling Biocatalytic Processes

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iocatalysis is an important technology for producing chiral chemical products and intermediates. But biocatalysts are sensitive, so control strategies are needed that rapidly monitor the changing concentrations of reactant and product. We used near-infrared (NIR) spectroscopy to monitor a biocatalytic process, which provided us with a control strategy that resulted in a 30% productivity gain. We designed and tested a monitoring and control strategy that uses a bioprocess model, ensuring a systematic approach that can overcome the product and reactant inhibition that often limits biocatalytic process development.

Baeyer–Villiger oxidation (including oxidation of a ketone, using peroxy acid) is an important method of producing lactones (chemically versatile anhydrides that are often used as key building blocks for industrially valuable targets).

Increased selectivity and byproduct prevention are some of the advantages of using biocatalytic processes for manufacturing chiral products and chemical intermediates. Yet no industrial processes are currently using biocatalysts with Baeyer–Villiger monooxygenases because, among other factors, available monitoring and control techniques have not been rapid enough to prevent reactant and product inhibition. However, as the authors describe, rapid monitoring and control can now be achieved using near-infrared spectroscopy that, during tests, resulted in a 30% gain in productivity.

Baeyer–Villiger oxidation in synthetic organic chemistry is achieved using a peracid, which makes any large-scale process risky because peracid preparation uses stoichiometric amounts of a peroxide (essentially 90% hydrogen peroxide) as a primary reactant. These bioprocessing difficulties are currently being addressed at several European laboratories as part of a multidisciplinary effort funded by the European Commission (EC, [http:// europa.eu.int/comm/index_en.htm](http://europa.eu.int/comm/index_en.htm)).

Lactones are particularly useful in biologically active products such as pharmaceuticals, agrochemicals, and flavor constituents. In recent years, researchers have been pursuing an answer to the question of how to use Baeyer–Villiger oxidation to produce optically pure products. One recent line of research that has achieved moderate selectivity involves reactions catalyzed by transition metals. But a more promising approach uses biocatalysis.

Biocatalysis: A Synthetic Tool

Biocatalysis uses enzymes to catalyze many chemical reactions. The enzymes used are either contained within microorganisms or in their pure (isolated) form. Enzymes are usually highly specific and selective, and it is those attributes that can be used to help prevent byproducts, simplifying the downstream processes.

Biocatalysis can also be used to carry out conversions that would otherwise require difficult or multiple synthetic chemical protection and deprotection steps. When optically pure products are desired, a biocatalyst can assist in regio- or stereochemistry. Optical purity using biocatalysis is usually achieved by either resolving a racemic mixture or by asymmetrically synthesizing a prochiral reactant. Both approaches are being studied for selective oxidation reactions, a process that is of particular interest to the pharmaceutical industry.

Using BVMOs. In selective oxidation reactions, an intact microorganism is often the biocatalyst. The use of whole cells as catalysts has the advantage of allowing cofactors, such as NAD(P)H, to be recycled in situ. *Baeyer–Villiger monooxygenases* (BVMOs) are redox enzymes capable of nucleophilic oxygenation of a wide range of linear or cyclic ketones, yielding

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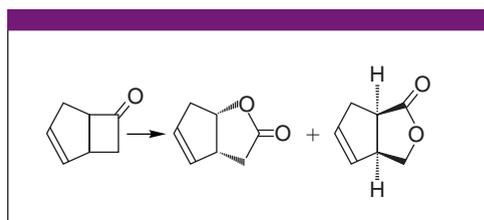


Figure 1. Oxidation of bicyclo[3.2.0]-2-en-6-one to its corresponding regioisomer lactones (–) (1*S*, 5*R*), 2-oxabicyclo[3.3.0]oct-6-en-3-one (ee=94%) and (–) (1*R*, 5*S*), 3-oxabicyclo[3.3.0]oct-6-en-2-one (ee=99%)

(optically pure) esters or lactones. Redox enzymes use molecular oxygen and reduced cofactor NAD(P)H as a reductant to carry out Baeyer–Villiger oxidations (1,2).

BVMO applications that synthesize natural products are well documented. Table 1 shows bioconversions of some key chiral synthons catalyzed using BVMOs. Although the production of lactones is of industrial interest, there are, currently, no industrial processes that use a BVMO-catalyzed step. The primary reason BVMO catalysis is not used is the low volumetric productivities that result from some of the bioprocessing effects, such as product or substrate inhibition and cofactor requirements.

Product and Reactant Inhibition

Our goal was to develop a process that overcame biocatalysis *inhibition* difficulties (inhibition is the process of the end product of a metabolic reaction inhibiting an enzyme involved in that reaction as the reaction starts again, thus breaking the reaction cycle), resulting in oxidation at useful levels of product and reactant. Of more than 30 BVMOs, cyclohexanone monooxygenase (CHMO) is the best characterized. In our work, CHMO was expressed heterologously in *Escherichia coli* TOP10 [pQR239] (1). In a previous paper and shown in Figure 1, we reported biocatalyst kinetics for the oxidation of bicyclo[3.2.0]hept-2-en-6-one to its corresponding regioisomeric lactones (–)(1*S*, 5*R*)-2-oxabicyclo[3.3.0]oct-6-en-3-one (ee=94%) and (–)(1*R*, 5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one (ee=99%) (3). But there is a key problem to be solved before using CHMO in industrial processes: low reactant and target production. That is, when reactant or product levels in the medium fall outside an optimal range — 0.2–0.4 g/L for ketone or above 4.5 g/L for lactone — CHMO oxygenation is ineffective; specific activity is very low.

Reactant and product inhibition in biocatalysis (Table 2) is often the constraint that prevents scaleable biocatalytic reactions from becoming a

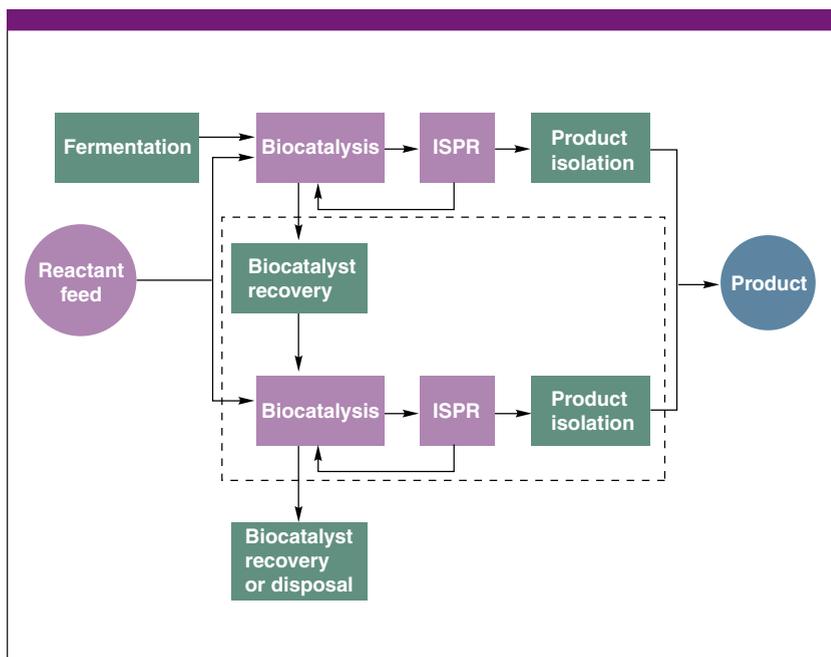


Figure 2. Efficient and productive operation requires that the addition of reactant be controlled and that an in-situ product removal process be in place. Monitoring of reactants and products (shown in purple) within biocatalytic reactors can improve operational control. Productivity can also be improved by maintaining biocatalytic activity, allowing the recovery and reuse of the biocatalyst.

Table 1. BVMOs are used to prepare a variety of natural products.

Compound	Application	Bioconversion details	Reference
Multifidene	Pesticide	Precursor synthesized from a bicycloketone using CHMO ^a	13
(<i>R</i>)-(-)-baclofen	Neurotransmitter derivative	Precursor synthesized from a substituted cyclobutanone	14
Sordidin	Pesticide	Precursor synthesized from a 2,6 disubstituted cyclohexanone	15
Azadirachtin	Insect antifeedant	Precursor synthesized from a bicycloketone using CHMO ^a	16
Sarkomycin A	Antibiotic	Precursor synthesized from a bicycloketone	17
Ionomycin	Antibiotic	Precursor synthesized using CHMO ^a	18
Carbocyclic nucleosides	Antiviral agents	Precursor synthesized from norbornanone using CHMO ^a	19

^aCHMO = cyclohexanone monooxygenase

Table 2. Biocatalyst examples exhibiting reactant and product inhibition

Reaction in Example	Biocatalyst in Example	Reference
<i>N</i> -acetyl-D-neuraminic acid (Neu5Ac)	Neu5Ac adolase	20, 21
Acrylonitrile conversion	Nitrile hydratase	22, 23, 24
Synthesis of D-malate	Maleate hydratase	25
Esterification of benzoic acid	Lipase	26
Carbon–carbon bond synthesis	Transketolase	4, 27, 28

Figure 3. A process map such as this one of a bioconversion depicts the cumulative effect on cyclohexanone monoxygenase (CHMO) activity at various levels of ketone and lactone in a Baeyer–Villiger oxidation reaction.

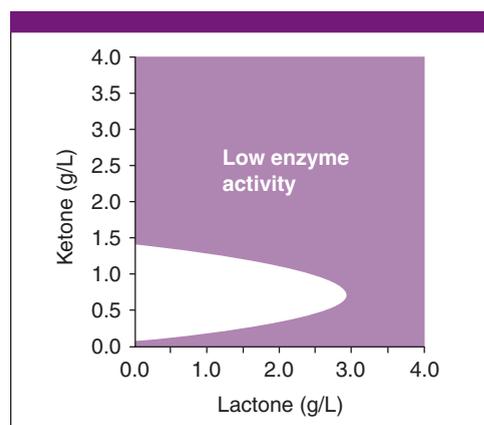
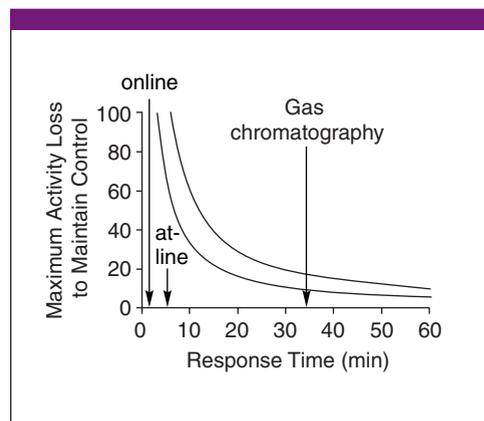


Figure 4. This plot depicts the time it takes to make a controlled change after a percentage drop in biocatalyst activity (assuming a ketone concentration of 0.5 g/L and a maximum control ketone threshold of 0.75 g/L).



standard method of carrying out complex syntheses. Lactone syntheses using whole cell Baeyer–Villiger catalysis are also restricted by product inhibition at low concentrations. To make biocatalytic scale-up efficient and productive, reactants must be fed into the bioreactor at a controlled rate that preserves optimal concentrations. And the lactone product must be removed from the reactor as it is formed. Figure 2 depicts such a scheme: Reactant is fed into a bioreactor in a way that prevents underfeeding as well as overfeeding. Similarly, lactone product is removed from the reactor using an in situ product removal technique (4).

Bioprocess maps. Bioprocess models of the cumulative effect of ketones and lactones on CHMO activity from Baeyer–Villiger reactions can be graphically represented in bioprocess maps (5). As Figure 3 shows, bioprocesses kept within defined areas of high enzyme activity can increase lactone yields (high CHMO activity is defined as the area of the process map in which enzyme activity is greater than 70% of the maximum activity after a specified time). Process maps are particularly useful in controlling bioprocesses, especially processes with a number of time-dependent variables, such as catalyst viability.

NIR monitoring. In a previous article, we showed that NIR spectroscopy permits sensitive and robust monitoring of analytes during a microbially catalyzed Baeyer–Villiger lactone synthesis (6).

To achieve those results, multivariate calibration is used to develop a spectral model that can predict results of the constituents we are interested in, such as reactant and product concentrations. Multivariate calibration of all NIR and reference data uses partial least squares (PLS).

Controlling the Process

Our goal was to find a method of controlling both ketones and lactones so that they could be maintained at levels that produce the highest enzyme activity.

Gas chromatography has traditionally been used to monitor ketone and lactone, but gas chromatography has a response time of 35 minutes (including sampling). Advanced process control techniques require a faster response, especially when monitoring areas of high CHMO activity or significant drops in enzyme activity (caused by ketone levels that drop below optimal concentration).

The flexibility of any control system depends on the time it takes to determine the new values of increasing or decreasing variables. Rapid monitoring is critical. In Figure 4, the ketone concentration in the reactor is 0.5 g/L with an upper threshold of 0.75 g/L, which promotes the highest CHMO activity (7). If CHMO activity is 23 mmol/h, the control system must respond within six minutes (before all catalyst activity is lost) when the ketone concentration rises above the control threshold (8). If biocatalyst activity is increased to 46 mmol/h, then the necessary response time drops to three minutes.

Both the addition and removal of the analytes require control of feedback loops, and that is only possible if rapid and quantitative monitoring of both the reactant and the product is in place. We describe a biocatalytic process that gives that type of rapid control. Using NIR spectroscopy for monitoring, our strategy enabled the biocatalyst to operate at useful reactant and product concentrations, resulting in a 30% productivity gain.

Testing the Production Methods

Our whole cell biocatalyst was produced in a 1.5-L fermentation reactor using a complex growth medium: 10 g/L of both glycerol and NaCl, 26 g/L of yeast extract, 13 g/L of tryptone peptone, and 50 mg/L of ampicillin. Growth medium constituents were obtained from Sigma-Aldrich (www.sigma-aldrich.com). Frozen stock culture (1 mL at -70°C) of *Escherichia coli* Top10 [pQR239] was donated by J.M. Ward (Biochemistry and Molecular Biology, UCL, www.biochem.ucl.ac.uk). The culture was thawed and inoculated into 1-L (0.1-L working volume) flasks, incubated at 37°C , and shaken overnight at 250 rpm. Then the culture was inoculated into an

LH210 series, 2-L (1.6-L working volume), stirred tank fermentor (Bioprocess Engineering Services, www.bioprocess-eng.co.uk). A pH of 7 (± 0.1) was maintained by adding 3 M NaOH and 3 M H_3PO_4 as required; the temperature was kept at 37 °C (± 0.1 °C).

The vessel was aerated with 0.67 v/v/m of air through a submerged sparger, and the impeller speed was adjusted to 600–900 rpm to maintain dissolved oxygen tension above 0%. The culture was grown until the optical density (o.d.) reached 10 at a wavelength of 670 nm, measured by a Kontron (www.kontron.com) Uvikon 922 variable wavelength spectrophotometer. To induce CHMO expression, we added 0.1 w/v L-arabinose. The culture was grown for an additional three hours until the o.d. at 670 nm reached 14–15. We have previously described the fermentation and scale-up of CHMO production and the kinetic characteristics of the whole cell catalyst (3,8).

Whole cell biocatalysis. Stereoselective oxidation of bicyclo(3.2.0) hept-2-en-6-one was carried out immediately after the fermentation of whole cell *E. coli* TOP10 [pQR239] and in the same reactor. The stirred-tank fermentor was used for all fed-batch biotransformations. We controlled pH at 7 (± 0.1) by adding 3 M NaOH and H_3PO_4 as required and kept the temperature at 37 °C (± 0.1 °C). We aerated the vessel with 1 v/v/m of air, using a submerged sparger and maintained impeller speed at 800 rpm throughout. Pure bicyclo[3.2.0] hept-2-en-6-one compound was continuously delivered to the biotransformation medium from an external reservoir using a peristaltic pump (Watson Marlow, www.watson-marlow.com). The medium was supplemented with 10 g/L glycerol before ketone was fed to the broth at 1.25 g/L/hr.

We removed 5-mL samples from the reactor at 10-minute intervals. The samples were immediately centrifuged for three minutes at 10,000 rpm (using a Heraeus Biofuge A Microfuge from Heraeus Instruments, www.heraeus-instruments.de) to remove the cells. The resulting supernatant liquid was analyzed using a fiber-optic transmission immersion probe with a model 6500 visible near-infrared (vis/NIR) spectrophotometer (FOSS NIRSystems, www.foss-nirsystems.com) with 32 averaged coadded spectrum scans. Vision software (FOSS NIRSystems) on a PC workstation managed the spectrum collection. To separate and quantify ketones and lactones, we used an autosystem XL-2S gas chromatograph (Perkin-Elmer, www.perkin-elmer.com) fitted with an Alltech (www.alltech-bio.com) series

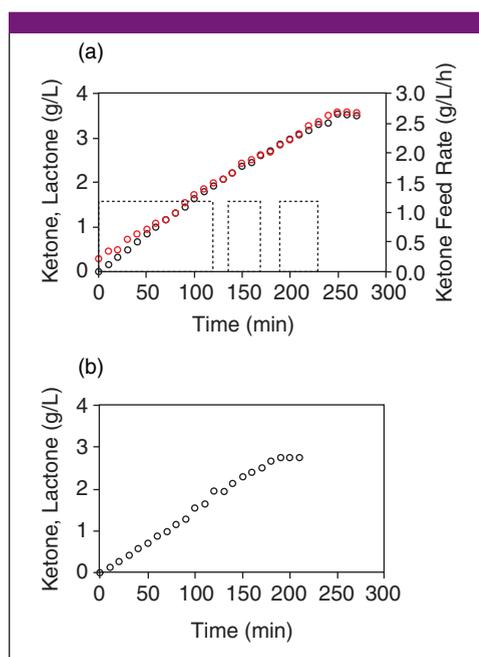


Figure 5. The profiles of a biotransformation process show lactone concentration as red and black circles. Ketone concentration is shown in the squares. The red circles are online NIR, and the dark circles are gas chromatography. In profile (a), ketone was fed into the reactor using a peristaltic pump and a NIR profile to control the feed rate. Profile (b) shows the response when ketone was continuously fed (1.5 g/L/h) into the reactor.

AT-1701 column (30 m \times 0.54 mm). Gas chromatography (GC) analysis was carried out after the ketones and lactones had been partitioned between the supernatant sample and the ethylacetate.

Building a Calibration Model

NIR spectroscopy is an optical technique that involves measurements between the visible (700 nm) and the mid-IR (2,200 nm) region. In quantitative NIR spectroscopy, empirical relationships (calibration models) are derived from the absorption (or transmission) of NIR radiation compared with corresponding reference assays.

We modeled second derivative (20-nm segments with gaps of 0 nm) relationships using partial least-squares regression (PLS) on the empirical relationship between spectra and analytical reference data. We used the 1,550–1,820 nm region of the electromagnetic spectrum for calibrations. PLS methods employ factor analysis then use a subset of the resulting factors to complete the regression equation. The best regression models cross-validate procedures to optimize the number of factors so that either a minimum prediction residual error sum of squares (PRESS) or a standard error of calibration (SEC) with the fewest factors can be used. Those data are similar to those associated with gas chromatography standard errors.

For data storage and reduction, we used Vision V2.22 software on a PC workstation. Using

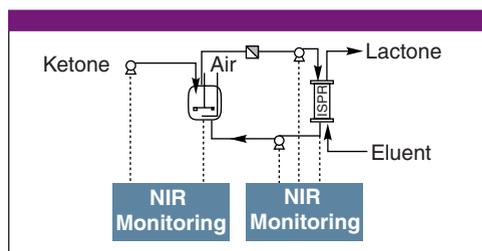


Figure 6. An optimum biotransformation requires that reactant and product be monitored at-line or online. Using NIR spectroscopy for monitoring enables feedback production strategies (shown as a dotted line) that control ketone (the feed) and lactone (through in-situ product removal).

regression models, we can make quantitative predictions of variables that come from unknown samples.

Analyzing the Data

PLS, a factor-based method (unlike multiple linear regression), does not require the user to prepare a calibration data set that includes all interfering species. This is especially important for bioprocesses, like the CHMO-mediated Baeyer–Villiger lactone synthesis, because the complexity of the reaction matrix (particularly from spent aqueous media of the preceding fermentation) leads to no detectable, distinctive, analyte spectral peaks.

By using characteristic samples (allowing for process variability), the calibration data set should, ideally, span the concentration range expected when routinely running the process. But without the ability to rapidly monitor fermentation, we were unable to control the ketone concentrations — and thus the lactone production — sufficiently to produce the desired lactone range in our calibration set. To provide

Table 3. Quantitative NIR calibration models were built for prediction of reactant ketone and the product lactone during a microbially catalyzed Baeyer–Villiger bioconversion. The ability of each model to predict unknown at-line samples taken from an independent biotransformation is indicated by the standard error of prediction (SEP).

Model	Concentration Range (g/L)	Number of Spectra in Model	SEP (g/L)
Lactone (1)	0–2.8	158	0.223
Lactone (2)	0–4.0	158+16 spiked	0.118
Ketone	<1.0	144	0.073

the missing data, we spiked some process samples with commercial lactone, a procedure we have described elsewhere (9).

We produced three at-line NIR calibration models, each containing spectra taken from seven bioprocess batches (Table 3). The lactone calibration model contained 16 spectra taken from samples spiked with commercial lactone, allowing the lactone concentration range to be extended from 2.8 g/L to 4.0 g/L (2). Because neither of the lactone regioisomers produced during stereoselective Baeyer–Villiger oxidation of bicyclo[3.2.0]hept-2-en-6-one were available commercially, (1*S*, 5*R*) 2-oxobicyclo[3.3.0]oct-6-en-3-one was used to spike the samples.

Monitoring results. We determined the usefulness of the monitoring system during a bioconversion by withdrawing samples every 10 minutes for NIR analysis. Monitoring allowed the ketone concentration to be maintained below the inhibitory levels defined by the bioprocess map. Reactant feed control was achieved by intermittent pump feeding (Figure 5a). The process with its monitoring and control resulted in 3.6 g/L of lactone, a 30% improvement in productivity when compared with previous continuous fed-batch operations (Figure 5b). Analysis of the process samples using the gas chromatography reference assay confirmed those results and enabled us to calculate a standard error of prediction for each NIR calibration model.

The ketone SEP (see Table 3) was sufficiently low (0.073 g/L) to allow the ketone concentration to be maintained within the 0.5–0.75 g/L threshold. The spiked lactone method resulted in a significantly lower SEP from that of the lactone model (1). Improved prediction with the spiked calibration model was, in part, a result of using a calibration data set that more closely represented the changes anticipated in the process matrix. Prediction of unknowns during monitoring are interpolated rather than extrapolated from the calibration model. Because multivariate calibration models characterize variance, variabilities during bioprocess development that differ from expectation require modifications to the calibration model. When a bioprocess map is used as a development tool, the calibration data set of the original model can be modified to reflect the difference. Figure 5 shows the effects of using the improved model for monitoring lactone.

Monitoring-Enabled Control

Synthesis of products with multiple chiral centers can use biocatalysis to circumvent the need for multiple, chemical protection and deprotection steps. Baeyer–Villiger oxidation has only

recently been achieved with transition metal chemistry and only with moderate selectivity (10). Bioconversions using high productivity and robust process strategies will enable biocatalysis to be exploited alongside conventional chemistry to produce highly complex, optically pure intermediates and products.

Biocatalytic optimizing. Rapid monitoring of a biocatalytic process has immediate advantages in controlling a bioprocess and results in increased productivity. Major advances in rapid process monitoring, however, will come from monitoring-enabled development of a bioprocess. For example, commercial bioprocess operations depend on maximum product yield from the biocatalyst. To achieve that, the whole cell catalyst must be used several times during its life. Therefore, it must be used under optimal kinetic conditions. Without rapid monitoring of the reactants and products enabling accelerated control, the bioprocess cannot achieve that efficiency.

In situ product removal. In addition to feedback control of ketones, lactones could be controlled within the optimum activity window of the bioprocess map (Figure 3) using in situ product removal (ISPR). ISPR allows product to be removed from the vicinity of the biocatalyst during a biocatalytic reaction. Figure 6 is a schematic that shows an ISPR column — separate from the reactor — through which reaction medium flows. A matrix with an affinity for the lactone enables removal of the product from the reaction stream so that the lactone concentration is maintained below the inhibitory concentration of 4.5 g/L.

Such an ISPR technique would require careful control of the column flow rates to maximize column loading and prevent breakthrough of product back into the stirred-tank reactor. Amberlite XAD4 (Rohm and Haas, www.rohmhaas.com) is a suitable affinity matrix for ISPR of the lactones in the example discussed here (11). Amberlite XAD4 applied to whole cell Baeyer–Villiger lactone synthesis resulted in an increase of final process product concentration. However, with that matrix, the ketone and lactone bind competitively to the column. That suggests that lactone yields during product recovery stages would be dependent on minimizing ketone concentration in the reaction stream. Such a holistic approach to process operation can be developed using rapid NIR monitoring during the biocatalytic step to direct the downstream processing.

Commercial biocatalysis for synthesizing novel compounds requires a robust and fast process development strategy. Such a systematic approach to biocatalytic process design is being

built, and acceleration of such technology integration can be achieved through increased application of process modeling (12). As we have described, the use and value of a process map and modeling to analyze process constraints can only be realized through advanced monitoring and control systems. **BPI**

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INFO +

Enbrel Hopes to Expand its Label

The battle of the arthritis drugs is heating up. Enbrel, one of the most successful biopharmaceutical treatments for rheumatoid arthritis (RA), will be challenged next year by Humira. Enbrel is a recombinant tumor necrosis factor (TNF) inhibitor, manufactured by **Immunex**

(www.immunex.com) but marketed by **Amgen** (www.amgen.com) and **Wyeth** (www.wyeth.com). Amgen and Wyeth have launched the second part of a 10,000-patient, phase 4 study known as RADIUS (Rheumatoid Arthritis DMARD Intervention and Utilization Study) that will compare Enbrel with other disease-modifying antirheumatic drugs (DMARDs). Enbrel is currently approved for psoriatic and rheumatoid arthritis, but Amgen and Wyeth have submitted a supplemental biologics license application to FDA in the hope that it will be approved to improve physical function in patients with moderately to severely active RA. Enbrel has been extremely successful for the two companies since FDA approval in 1998. Sales are expected to be \$800 million this year, and demand for it is so high that around 30,000 patients remain on a waiting list. More than 129,000 patients have already been treated with the drug.

Abbott Laboratories (www.abbott.com) wants a piece of that pie. Its experimental TNF inhibitor, Humira (also known as D2E7), could be a significant competitor to Enbrel. Not only has Humira — expected to be approved early in 2003 — proved safe and effective in late-stage clinical trials, it only needs to be self-injected twice a month instead of twice a week like Enbrel, and may be cheaper. Amgen is currently conducting another study evaluating whether Enbrel can be administered once a week. If it can, then Humira's convenience advantage will be significantly lessened. Amgen denies that the study is related to Abbott's likely entry into the TNF inhibitor market next year.

Industry News

DNA Sciences will collaborate with **Merck KGaA** to identify DNA variants that may modify responses to medications.

Microscience formed a joint venture with **Evotec OAI** called **Vmax** that will develop new types of antimicrobials.

King Pharmaceuticals will acquire **Meridian Medical Technologies** for \$247 million in stock. **Xerion Pharmaceuticals** and **Arius Research** will collaborate to develop colon cancer drugs. **Evogene** will collaborate with **Metabogal** to develop a platform that will produce therapeutic recombinant proteins from plants. **ChondroGene** will collaborate with **Pfizer** to identify novel therapeutic targets and biomarkers for treatment and diagnosis of osteoarthritis. **Inhale Therapeutic Systems** has signed an agreement to develop a PEGylated version of **InterMune's** chronic hepatitis C drug, **Infergen**. **Invitrogen** will acquire **InforMax** for \$42 million in cash.

Serono S.A. will collaborate with **Cellular Genomics** to apply chemical genetics technologies to kinases. **GlaxoSmithKline and Biosynex** will develop and commercialize staphylococcus antibodies and vaccines, including **BioSynex's** lead MAb BSYX-A110, which is currently in development. **Celgene** has acquired **Anthrogenesis**.