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Near-IR Spectroscopic Monitoring of Analytes during Microbially Catalysed Baeyer–Villiger Bioconversions

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Abstract:

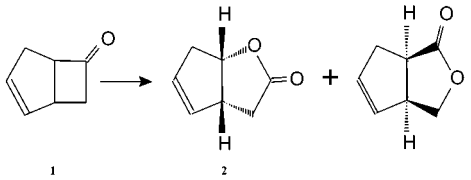
Sensitive and robust monitoring of product and reactants in a complex bioconversion stream is essential for the development of effective process-control strategies. In this contribution we report the use of near-infrared spectroscopy (at-line and on-line) to monitor a microbially catalysed Baeyer–Villiger bioconversion of a cyclic ketone to an optically pure lactone. The cyclohexanone monooxygenase-catalysed reaction is characterised by substrate (ketone) and product (lactone) inhibition of enzyme activity at relatively low concentrations. Quantitative multivariate calibration of a near-IR spectrophotometer for ketone and lactone resulted in a standard error of prediction at-line of 0.088 and 0.110 g/L and on-line of 0.130 and 0.180 g/L, respectively. The concentrations of substrate and product could be simultaneously monitored by near-IR, which had a response time of 5.0 and 0.75 min at-line and on-line, respectively. This work has indicated that near-IR spectroscopy has the potential to permit the realisation of an improved control strategy for this conversion based on these response times.

Introduction

Biocatalysts are increasingly used during the production of industrially significant compounds.[1–3] Novel, optically pure products can be synthesised efficiently in a single-unit operation by means of biocatalysis due to the characteristically good chemoselectivity, regioselectivity, and stereoselectivity of enzymes. Where an alternative chemical conversion is also available for product synthesis, a greater number of reaction steps are typically required.[2] In addition, reaction conditions are often aqueous based, requiring mild operating temperatures and pressures. Thus, a bioprocess can have convincing advantages with respect to process economics and the environmental impact when compared to those of chemical conversions. However, under operating conditions, the substrates and products are frequently toxic or inhibitory to the biocatalyst. This necessitates the use of advanced control systems[4] requiring process monitoring of the analytes of interest.

The Baeyer–Villiger flavoenzyme cyclohexanone monooxygenase (CHMO) catalyses the nucleophilic oxygenation

Scheme 1. **Stereoselective oxidation of bicyclo(3.2.0)hept-2-en-6-one to yield to (−)-1(*S*),5(*R*)-2-oxabicyclo(3.3.0)oct-6-en-3one and (−)-1(*R*),5(*S*)-3-oxabicyclo(3.3.0)oct-6-en-2-one**



of a wide variety of linear or cyclic ketones to corresponding optically pure esters and ketones.[5] Optically pure chiral synthons produced via CHMO could be used for the synthesis of a variety of value-added fine chemicals and intermediate pharmaceuticals. Here the stereoselective oxidation of bicyclo-(3.2.0)hept-2-en-6-one (**1**) to its corresponding regioisomeric lactones (−)-1(*S*),5(*R*)2-oxabicyclo(3.3.0)oct-6-en-3one (**2**) (ee = 94%) and (−)-1(*R*),5(*S*) 3-oxabicyclo(3.3.0)oct-6-en-2-one (**3**) (ee = 99%) is achieved (Scheme 1) by a recombinant whole cell biocatalyst using *Escherichia coli* expressing CHMO. This reaction has been reported previously.[6,7] The fed-batch biotransformation was undertaken, in the broth of the preceding fermentation, using an agitated stirred tank.

Under typical bioconversion operating concentrations, the substrates and products are toxic[4] or inhibitory to the bioctalyst.[7] The use of a fed-batch stirred tank reactor with feedback control enables the substrate concentration to be maintained in the optimum range. Such a system typically requires monitoring of the substrate at low concentration whilst maintaining a minimum sampling and analysis time. Where a bioconversion is characterised by product inhibition, then strategies to circumvent the problem can be adopted,[8,9] or the process can be run to maximize productivity according to the enzyme kinetics. In a previous paper[6,7] we have described the kinetics of the whole-cell *E. coli* Baeyer–Villiger bioconverson of ketone **1** to its corresponding regioisomeric lactones **2** and **3**. The optimum specific activity of 55 μmol/min/g was observed at a ketone concentration between 0.2 and 0.4 g/L, whilst at 5 g/L this was reduced by more than 90%. In addition when the combined lactone

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concentration was greater than 4.5 g/L the specific activity was zero. It is clear that control and optimisation of this bioconversion would be significantly improved by an effective on-line or rapid at-line monitoring technique.

Conventionally the monitoring of biochemical constituents has been conducted off-line, using laboratory methods such as gas chromatography and HPLC. The time-consuming nature of chromatographic methods (typical analysis time 10−40 min), and the requirement for sample preparation means these methods are not always suitable for process-control operations. Near-IR spectroscopy is a rapid, stable and nondestructive technique, requiring no sample preparation and capable of monitoring multiple components of a bioprocess in situ with an optical-fibre probe. Near-IR, is therefore especially useful when the removal of a sample from a reactor is problematic, for example for reasons of operational toxicity or sterility or where remote location of equipment is an advantage. The application of near-IR (NIR) to a wide range of analytical applications is testament to the interest that has been shown in the method.

Numerous NIR spectroscopic methods have been developed for fermentation processes including fermentations of yeast,[10−12] bacteria,[13−17] animal cells,[18−20] and insect cells.[21,22] Wider bioprocess applications include the monitoring and control of a down-stream yeast cell homogenate.[23] There has been no study of the application of NIR spectroscopy to biocatalysis, although real-time monitoring of nitrile bioconversions by mid-IR spectroscopy[24] has demonstrated the potential of vibrational spectroscopy for the study of biocatalysis.

The absorption of NIR rays in the 700−2500 nm region (14000−4000 cm^{-1}) is related to the overtone and combination bands of the fundamental infrared vibrations of the −CH, −NH, and −OH molecular groups. The method is therefore suitable for monitoring any bioconversion where the constituents of interest are organic molecules. Mid-IR absorption at fundamental frequencies (1500−400 cm^{-1}) are typically 10−100 times stronger than NIR[25−26] and capable of providing distinct spectral features of a compound from a mixture.[27] Mid-IR in situ analysis of aqueous mixtures using attenuated total reflection has been demonstrated.[27,28] The mid-IR system uses optical conduit technology containing knuckles with internal mirrors as a means of guiding the radiation between the sensor and the spectrophotometer. Since spectral features are broad and overlapped in the NIR region, resolution of spectral data for analyte calibration therefore requires chemometric techniques for multivariate analysis. In addition water strongly absorbs IR radiation, exhibiting strong OH bands at 1450 and 1940 nm, making extraction of quantitative information from the NIR spectra more difficult. However, the weak and broad features of absorption bands of NIR when applied to complex aqueous-based mixtures are an advantage since no sample preparation or dilution is required. NIR methods use bundles of flexible optical fibres, such that the spectrophotometer can be remotely situated some distance from the reactor. Since the NIR technique is rapid and requires no reagents for analysis and the instrumentation can be located some distance from the reactor site, there is a real potential to improve bioprocess operation in the chemical and pharmaceutical industries by monitoring conversions with NIR spectroscopy.

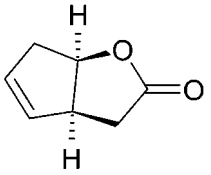

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Figure 1.

This study reports the first example of the application of fibre-optic NIR spectroscopy for the monitoring of analytes at low concentrations during a whole-cell *E. coli* bioconversion. Calibration for substrate and product concentrations both at-line and on-line is discussed.

Results and Discussion

NIR Spectra. The NIR spectra of ketone **1**, 1(*R*),5(*S*)-2-oxobicyclo(3.3.0)oct-6en-3one (**4**) (Figure 1) and the solvent (water) are depicted in Figure 2. Lactone **4** is commercially available, but lactones **2** and **3** are not. Lactone **4** was therefore used during feasibility analysis to locate wavelength regions for calibration of the ketone and lactone. As the regioisomers **2** and **3** are distinct molecules with different physical properties, individual IR vibrational signatures would be expected. However, because the regioisomeric disparity is only the site of oxygenation on the cyclic ketone[29] no distinction of these forms was made during NIR analysis. The NIR region contains first-order, second-order, and combinations of fundamental vibrations occurring in the mid-IR region, resulting in broad overlapped spectral bands such that the spectra of lactone **4** were able to provide a

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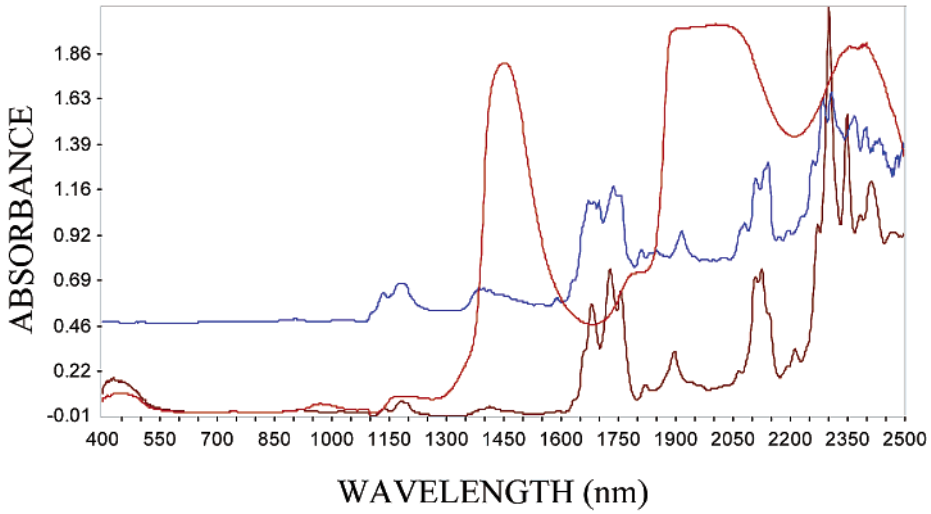

Figure 2. **Raw NIR spectra of lactone (blue) and ketone (brown) compared to water**

good indication of the wavelength region for combined lactones **2** and **3** calibration.

The spectra for ketone **1** and lactone **4** are similar with significant peaks in the first overtone region 1666 nm, 1750, 1900, and 1950 nm. The combination band region also contains peaks including 2150 nm and the region 2250−2300 nm. The majority of the vibrations resulting from CH−CH_2 bond stretches are common to both the ketone and the lactones. It was therefore apparent that selection of unique spectral features for calibration was not possible. A multivariate partial least-squares (PLS) method was therefore used. Distinctive peaks of water at 1450 and 1950 nm limit the regions for calibration. In addition, the use of fibre optics to facilitate on-line monitoring limits the calibration region further as the fibre-optic cable absorbs NIR radiation above 2200 nm.

NIR Sampling. The bioconversion medium consisted of the undefined medium of the preceding *E. coli* fermentation with the batch addition of glycerol and fed-batch addition of ketone. Such a bioprocess sample is a highly complex NIR matrix containing numerous metabolites. The on-line sampling environment also included biomass and sparged air, both of which cause scattering of NIR radiation. Baseline differences between spectra can result from scattering of radiation. Conversion of spectra to the second derivative reduced these effects.[14] As spectra were presented as a ratio to air background (despite background spectral information being predominately due to water) the second derivative calculation also served to enhance weak spectral features. A common procedure both in the application of near- and mid-IR is to remove background (water) information such that spectral information attributable to the constituents of interest is not swamped by bands associated with the solvent. However, the spectrophotometer was more precise when a spectrum ratioed to air rather than to water was applied. The standard deviation of intensity values between second-derivative spectra at 10 wavelengths in the region of 1500−1700 nm was 0.00559 for spectra ratioed to air and 34.4276 intensity units for spectra ratioed to water. The substantial increase in precision when spectra were ratioed to air can be explained by the fact that air is a stable background matrix compared to water.

Pollard and co-workers[27] presented their mid-IR sample spectra as a ratio to water, as this was the major solvent in the fermentation broth. They were able to achieve very good precision using the major absorber as a background. The use of air as a background for the weaker NIR absorbance allows for increased instrument sensitivity. A major requirement of the background is stability. The background should be the same every time to reduce the addition of noise to the spectral data. The spectrum of water as it exists in the sample is likely to be not the same as pure water, due to the change attendant upon the interactions of the water molecules with the solute. Despite sodium chloride having no absorption bands whatsoever in the NIR, Hirschfeld[30] was able to measure the salinity of seawater indirectly by the shifts induced on the water bands by the dissolved salts.[30]

Calibration of NIR spectra to a gas chromatography method using air as a reference resulted in standard errors of prediction (SEP) at submicromolar levels.

Calibration. PLS calibration was achieved for both on-line and at-line data sets. Production of the whole-cell *E. coli* biocatalyst[6,31] was by way of fermentation prior to the bioconversion. The Baeyer−Villiger reaction was undertaken in the fermentation broth. Any batch variation from the fermentation step was transferred to the subsequent bioconversion and resulted in increased batch-to-batch variation in the bioconversion step. Variation between three separate bioconversions was included in the calibrations, which incorporated 52−53 spectra taken both at-line and on-line. Calibration models were built for ketone and lactone using a PLS method. Predicted residual error sum of squares (PRESS) analysis was used to optimise the equations for number of principle component factors.

Calibration of predicted and measured (gas chromatography) concentrations for lactone and ketone using data taken on-line from three bioconversions resulted in standard errors

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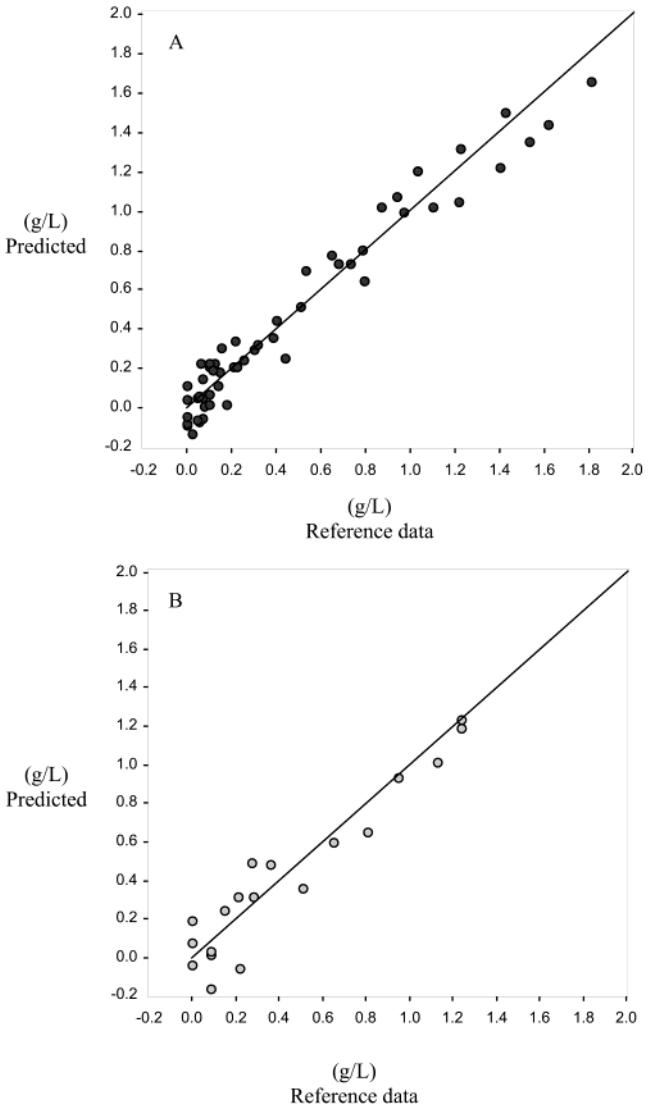

Figure 3. **Concentration correlation plots. (A) On-line ketone calibration eq A. SEC = 0.111 g/L. (B) Prediction of unseen on-line data using eq A. SEP = 0.130 g/L, external validation method.**

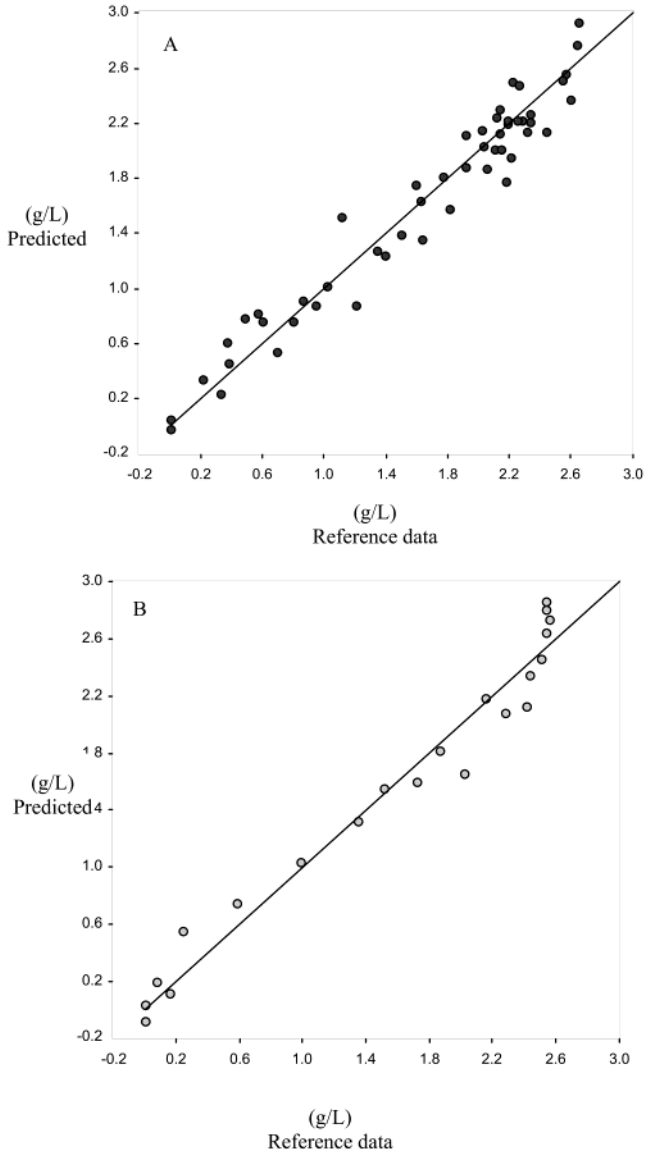

Figure 4. **Concentration correlation plots. (A) On-line lactone calibration eq B. SEP = 0.182 g/L (B) Prediction of unseen on-line data using eq B. SEP = 0.177 g/L, external validation method.**

of calibration (SEC); eq A, 0.111 g/L (1.02 mM) ketone (Figure 3) and eq B, 0.182 g/L (1.452 mM) lactone (Figure 4). Validating the on-line equations with a fourth, independent sample data set revealed SEP of 0.130 g/L (1.200 mM) ketone (Figure 3) and 0.177 g/L (1.425 mM) (Figure 4) lactone. The on-line method involved immersing the transmission probe directly into the reactor via the head plate. Each spectra consisted of 64 co-added scans and took 45 s to sample. The at-line method scanning time was only 30 s as the spectra consisted of only 34 co-added scans. However, the withdrawal of samples from the reactor and removal of biomass by centrifugation meant a routine sampling time of 5 min was achieved. The SEC of at-line spectra to gas chromatography data was significantly lower than on-line values with eq C, 0.074 g/L (0.689 mM) ketone (Figure 5) and eq D, 0.119 g/L (0.958 mM) lactone (Figure 6). Validation of at-line equations for ketone and lactone resulted in SEP of 0.088 g/L for ketone and 0.101 g/L for lactone.

Figure 7 on-line and Figure 8 at-line compare the predicted and measured concentration profiles for ketone and lactone. The calibration methods are outlined in Table 1. The SEP is considered a reliable measure of performance of the calibration equation, but should be considered alongside the multiple correlation coefficient (r^2). The correlation coefficient indicates a measure of the lack of error of the calibration, and for all the equations the r^2 values were greater than 0.95, indicating >95% of the variation within the data was modelled. At-line models gave correlation coefficients closer to unity (approximately 0.98) than on-line models. On-line monitoring included biomass agitation and aeration which cause light scattering that produces baseline offsets,[32] a source of variation explaining the higher SEP values and lower r^2 values. The robustness of the at-line system is further exemplified by the disparity between on-line and at-line *F*-values (Table 1). *F*-values give an indication of how well the accuracy of the calibration model (SEC) can be expected

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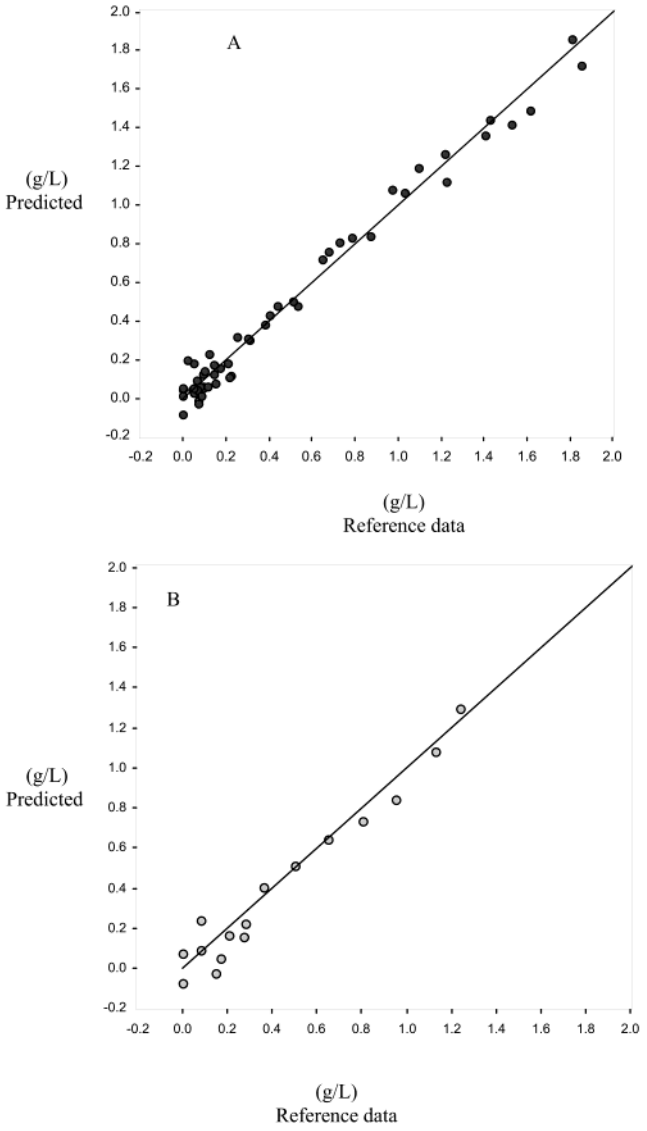

Figure 5. **Concentration correlation plots. (A). At-line ketone calibration eq C. SEP = 0.074 g/L. (B). Prediction of unseen at-line data using eq C. SEP = 0.088 g/L, external validation method.**

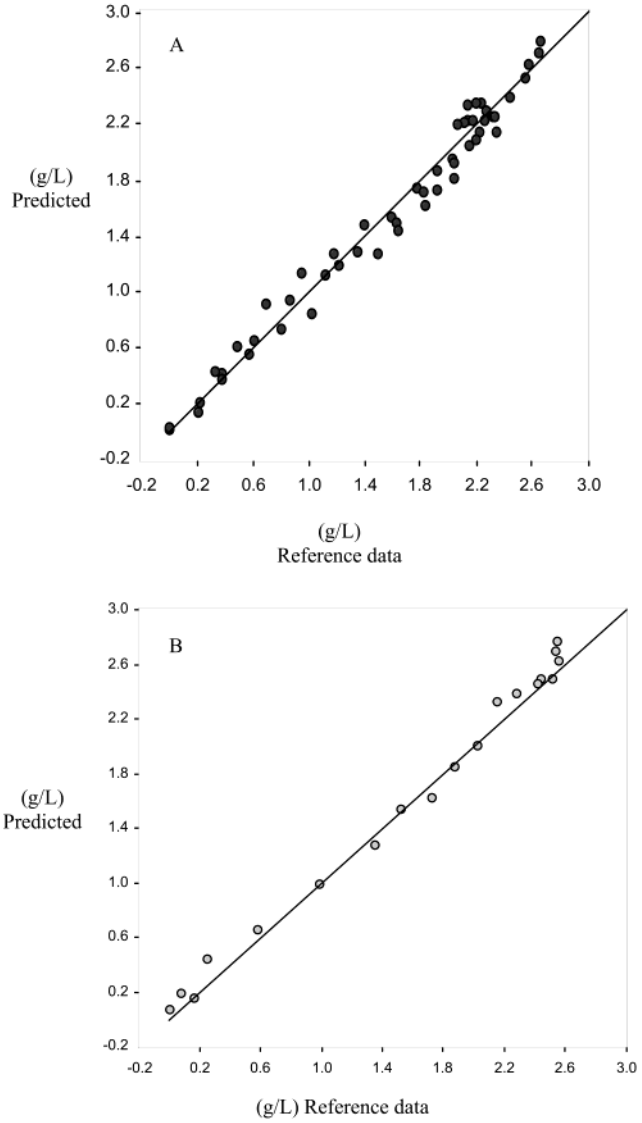

Figure 6. **Concentration correlation plots. (A) At-line lactone calibration eq D. SEP = 0.119 g/L. (B) Prediction of unseen at-line data using eq D. SEP = 0.110 g/L, external validation method.**

to hold up when unknown samples not included in the calibration are measured.

Independent validation of eqs A−D by predicting unknown samples independent to the calibration sets confirmed that the at-line NIR system is more accurate, as expected, at predicting values than the on-line method (Table 2).

Major disadvantages of at-line models include the increased response time (5 min) and the requirement for sampling of the reactor. A decision on the sampling method for NIR analysis of a bioconversion would have to include control requirements, such as predictive tolerance, and the response time required to maintain control within defined threshold values. There is an interest here to monitor the ketone and lactone at low concentrations so that strategies can be implemented to remove the effect of inhibition of the biocatalyst by the reactants. The at-line method would allow process monitoring at submillimolar levels. Future work is required to investigate the utility of NIR monitoring for controlling the whole-cell bioconversion.

This work has indicated that NIR spectroscopy can be applied to monitoring whole-cell biocatalytic reactions at-line and on-line. The application of a technique to monitor analytes at low concentration demonstrates the utility of NIR for control of bioconversion processes, which are characterised by substrates or products that are inhibitory or toxic or both. At-line sampling was significantly quicker than the gas chromatography technique used to monitor the Baeyer−Villiger reaction and is accurate enough to detect millimolar quantities of the substrate and product. The ease of rapid monitoring on-line provides the potential for intelligent control strategies to be applied to bioprocesses with rapid reaction rates.

Experimental Section

Materials. To aid wavelength region selection raw NIR spectra of ketone **1** and lactone **4** kindly donated by Fluka Chemie AG (Buchs, Switzerland) were analysed using transmission and reflectance spectroscopy, respectively.

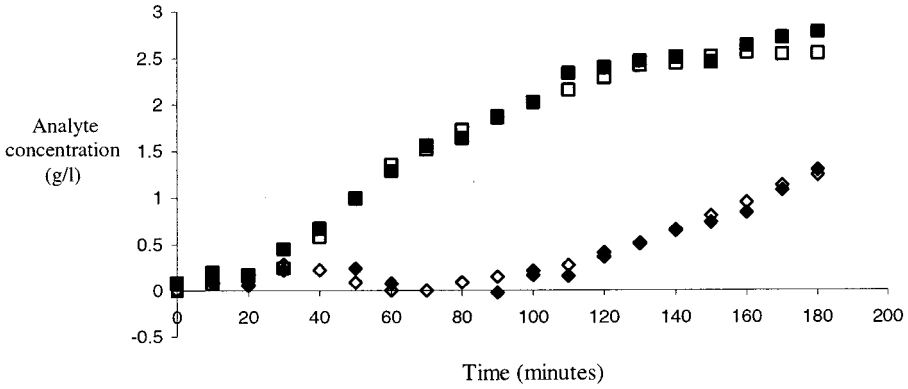

Figure 7. **1.5 L fed batch microbially catalysed Baeyer−Villiger bioconversion profile: Lactone: (■) on-line NIR, (□) gas chromatography. Ketone: (◆) on-line NIR, (◇) gas chromatography.**

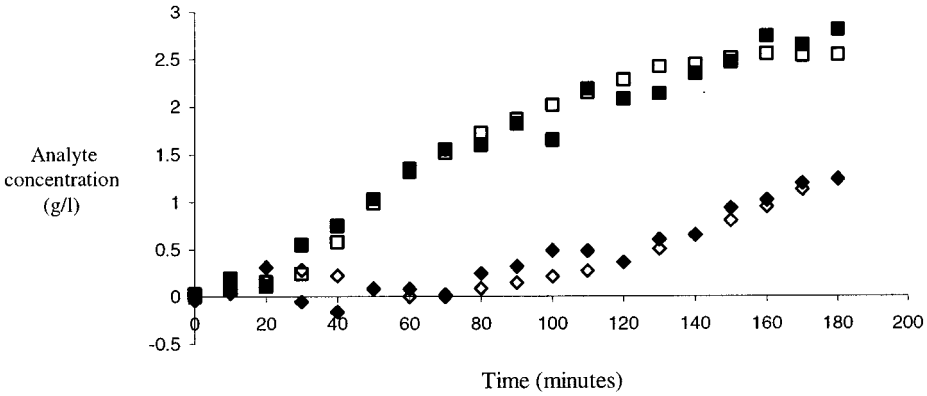

Figure 8. **1.5 L fed batch microbially catalysed Baeyer−Villiger bioconversion profile: Lactone: (■) at-line NIR, (□) gas chromatography. Ketone: (◆) NIR, (◇) gas chromatography.**

Table 1. **Calibration of NIR spectra to gas chromatography data[a]**

eq	analyte	*n* calibration	wavelength (nm)	factors	r^2	SEC (g/L)	F
A	ketone	53	1510−1800	4	0.96	0.111	254
B	lactone	52	1536−1820	4	0.95	0.182	240
C	ketone	52	1590−1800	5	0.98	0.074	522
D	lactone	52	1550−1820	3	0.98	0.119	728

[a] All spectral data were mathematically treated; second derivative before PLS regression to build on-line eqs A and B, and at-line eqs C and D. Three independent Baeyer−Villiger bioconversion data sets were used to build equations. A fourth set was used to perform an external independent validation (Table 2).

Table 2. **Validation of NIR calibration models was performed using an external validation set that was independent of the calibration set**

eq	sampling method	*n* validation	SEP (g/L)	r^2
A	on-line	20	0.13	0.9073
B	on-line	21	0.18	0.9669
C	at-line	16	0.09	0.9573
D	at-line	19	0.11	0.9913

Lactone **4** was used since the products of the stereoselective oxidation of ketone **1** by *E. coli* TOP10 [pQR239], lactones **2** and **3** were not commercially available.

Growth media constituents; glycerol, yeast extract, NaCl, and tryptone used for the *E. coli* TOP10 [pQR239] fermentation were obtained from Sigma Chemical Co. (Poole, Dorset, UK).

Fermentation. The cloning of CHMO from *Acinetobacter calcoaceticus* NC1MB 9871 to *E. coli*,[33] fermentation for CHMO production,[6] and the kinetic characteristics of the whole-cell catalyst, *E. coli* TOP10 [pQR239] have been reported previously.[7] Whole cell biocatalysts were produced in 1.5-L fermentations using complex growth media (10 $g{\cdot}L^{-1}$ each of glycerol and NaCl, 26 $g{\cdot}L^{-1}$ yeast extract, 13 $g{\cdot}L^{-1}$ tryptone peptone, and 50 $mg{\cdot}L^{-1}$ ampicillin). One milliliter of frozen stock cultures (−70 °C) were thawed and inoculated into 1-L shaken flasks (working volume, 0.1 L) and incubated at 37 °C overnight with shaking (250 rpm). This culture was then inoculated into a LH210 series 2-L stirred tank fermenter (working volume, 1.6 L) (Bioprocess Engineering Services, Charing, Kent, UK), where the pH was maintained at 7 (±0.5) via the addition 3 M NaOH and 3 M H_3PO_4, and the temperature at 37 °C (±0.5 °C). The vessel was aerated with 0.67 vvm air via a submerged sparger, and the impeller speed maintained at 600 rpm throughout. The culture was grown until the optical density (at 670 nm), measured using a Uvikon 922 variable wavelength spectrophotometer (Kontron Watford, Herts, UK), reached 8 (approximately 4 gdcw/L) after which 0.1 w/v L-arabinose was added to induce CHMO expression. The culture was grown for a further 3 h until the OD at 670 nm reached 12 (approximately 6 gdcw/L).

Whole-Cell Bioconversion. The stereoselective oxidation of ketone **1** by whole-cell *E. coli* TOP10 [pQR239] was

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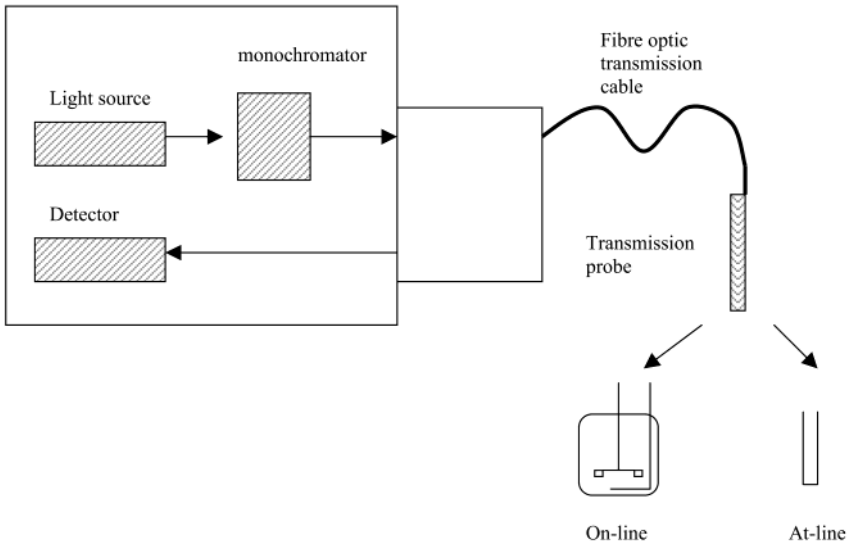

Figure 9. **NIR system for monitoring the bioconversion.**

carried out immediately after and in the same reactor as the fermentation step. The LH 210 series 2-L (working volume 1.6-L) stirred tank fermenter was used for all fed-batch biotransformations. The pH was controlled at 7 (±0.5) via the addition 3 M NaOH and 3 M H_3PO_4, and the temperature, at 37 °C (±0.5 °C). The vessel was aerated with 1 vvm air via a submerged sparger, and the impeller speed was maintained at 800 rpm throughout. Ketone **1** was continuously delivered to the bioconversion media as a pure compound neat from an external reservoir via a peristaltic pump (Watson-Marlow, Falmouth, Cornwall, UK). The media was supplemented with 10 $g \cdot L^{-1}$ glycerol prior to feeding ketone at 1.5 $g \cdot L^{-1} \cdot h^{-1}$.

Reference Analysis. Samples taken from the reactor at regular intervals (10 min) were immediately centrifuged (2 min) at 10 000 rpm (Heraeus Biofuge A Microfuge, Heraeus Instruments, Newtown, CT) to remove the cells. The separation and quantification of ketones and lactones were performed using a Perkin-Elmer autosystem XL-2 gas chromatograph (Perkin-Elmer, Norwalk, CT) fitted with an Alltech series AT-1701 column (30 m × 0.53 mm) (Alltech, Carnforth, Lancs, UK) which was packed with 14% cyanopropylphenyl and 86% methylpolysiloxane. Nitrogen carrier gas was supplied at 4 $mL \cdot min^{-1}$. The GC temperature programme used was 100 °C held for 5 min and then ramped at 10 °C min^{-1} until it reached 240 °C. Ketone and lactone concentrations were determined from an external calibration curve.

NIR Analysis. A fibre-optic transmission immersion probe (FOSS UK Ltd, Warrington, Cheshire, UK), with a total path length of 1.5 mm was used for all at-line and on-line sample analysis. During on-line work the probe fitted to the head-plate of the reactor through a 20-mm port and was situated so that the cell path was 3.0 cm from the reactor base. Spectra of the raw lactone were ratioed to a ceramic background using a Foss NIR systems reflectance probe. A Foss NIR systems model 6500 vis/NIR spectrophotometer equipped with a halographic concave diffraction grating and a lead sulfide (PbS) detector was used to measure the spectra (Figure 9). Vision software (Foss UK Ltd., UK) was used with a PC workstation to control spectra collection. On-line samples were ratioed to air background at regular intervals using 64 coaveraged scans per sample throughout the bioconversion process. At-line sampling required a 5-mL volume of broth being removed from the reactor at regular intervals and immediately centrifuged at 10 000 rpm (2 min) before NIR spectra (with air as background) were recorded. Samples at-line consisted of 32 coaveraged scans.

Multivariate Calibration. Vision 2.22 (Foss UK Ltd., UK) software was used in conjunction with a PC workstation for the storage and reduction of all data. Calibration between second derivative mathematically treated spectra and associated reference analytical data were produced using a partial least-squares (PLS) approach. The mathematical treatment to obtain second derivatives for the sample spectra used a segment size of 20 nm and gap size of 0 nm. The PLS method employs factor analysis and subsequently uses a subset of the resulting factors to complete the regression analysis. The initial equations for ketone and lactone were based on all factors contributing to the spectral data. The standard error of calibration (SEC) was shown with all calibration equations. The SEC indicates the standard deviation between residual values, the errors, between the predicted and reference measured values. The best possible regression equations were achieved by a cross-validation procedure optimising the number of factors such that either a minimum prediction residual error sum of squares (PRESS) is reached or the fewest factors required to achieve a standard error of calibration (SEC) similar to the associated gas chromatography standard error are obtained. Cross validation involved each calibration sample being dropped out of the calibration set, generating regression equations on the reduced calibration set and then subsequently predicting the value of the dropped standard.[34] Validation of calibration equations was achieved using a spectral sample set that was independent of the calibration set.

(34) Chalmers, J. M.; Dent, G. *RSC Analytical Spectroscopy Monographs*; Royal Society of Chemistry, UK, 1997.

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