New Frontiers in Screening for Microbial Biocatalysts
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A DRASTIC (DIFFUSE REFLECTANCE ABSORBANCE SPECTROSCOPY TAKING IN CHEMOMETRICS) Approach for the Rapid Analysis of Microbial Fermentation Products: Quantification of Aristeromycin and Neplanocin A in *Streptomyces citricolor* Broths.

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Microbial cultures can provide metabolites which are useful as structural templates for rational drug design. Increasing the titre of the metabolite is an important part of this process and is often achieved by random mutagenesis. As titre-improved mutants derived by this method are extremely rare, many thousands need to be screened. Screening mutants for increased metabolite production relies on methods such as assessing binding *via* the scintillation proximity assay or identifying an increase in concentration using chromatography. Such methods are typically restricted by the necessity to perform solvent extractions and, in the case of HPLC analysis, to optimise separation of the components of interest. Although the routine procedures can be automated, such multi-step screening processes are far from ideal.

Diffuse reflectance absorbance infra-red spectroscopy provides an alternative rapid, automated, quantitative approach which yields more detailed information about chemical characteristics than, for example, the UV absorbance spectrum typically used in HPLC analysis. The method can also be employed non-invasively on unprocessed fermentation samples.

We demonstrate the use of this spectroscopic technique in combination with chemometrics for determining the concentrations of aristeromycin and neplanocin A in *Streptomyces citricolor* fermentations. The fermentation broths of a range of mutants previously obtained during a titre improvement programme were analysed by standard HPLC techniques and by automated diffuse reflectance absorbance infra-red spectroscopy. Chemometric processing of the infra-red spectra was performed using supervised and unsupervised multivariate calibration methods. DRASTIC proved to be a rapid and reliable method for the estimation of metabolite overproduction in cultures of biotechnological interest, and it was possible to discriminate cultures overproducing closely related molecules.

1. INTRODUCTION

High throughput screening (HTS) in drug discovery programmes allows many thousands of biological and chemical samples to be tested against any number of disease targets. Natural products active in these screens are often present at very low levels and their overproduction is therefore critical for further analysis. With microbial metabolites the yield is often increased by generating mutant strains that accumulate the metabolite of interest. Large numbers of cultures need to be tested to isolate overproducers, with throughput limited by the time required for the metabolite assay, which is usually performed by HPLC. In fact, although details are understandably difficult to come by, it would appear that a great many promising metabolites fail to proceed to clinical trials exactly because of the difficulty of making them in sufficient quantities. DRIFT (Diffuse Reflectance Infra-red Fourier Transform) spectroscopy, which we have previously shown can be used to give an accurate and precise estimation of the concentration of ampicillin added to an *Escherichia coli* cell background [1] (see also Kell *et al.*, this volume), is a novel, rapid and largely non-destructive alternative to such screening methods.

1.1 *Streptomyces citricolor* Model Fermentation System

Aristeromycin and neplanocin A (Figure 1) are naturally occurring carbocyclic nucleosides produced by *Streptomyces citricolor* (reviewed in [2]). Both molecules have a close structural similarity to adenosine and possess potent biological activity, including inhibition of *S*-adenosylhomocysteine hydrolase [3]. Neplanocin A also exhibits anti-tumour [4] and anti-viral activity [3]. Analysis of the biosynthetic pathway using blocked mutants has shown that neplanocin A is a precursor of aristeromycin [2, 5]. Isolation and characterisation of such mutants is a key feature of strain development programmes aimed at final product or intermediate metabolite overproduction. Screening of potentially useful mutants after chemical mutagenesis typically requires a multistep process involving HPLC separation of the medium components. Although the rapidity of these techniques can be increased by automation, throughput is significantly affected by the physical limitations of HPLC separation. In this study we demonstrate how rapid, quantitative screening of fermentation products by infra-red spectroscopy using the DRASTIC approach can be used to identify *S. citricolor* mutants which overproduce aristeromycin and/or neplanocin A.

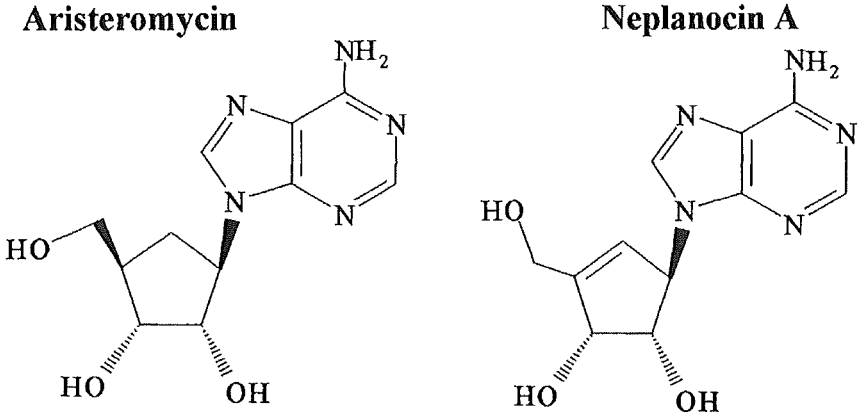

Figure 1. Structures of Aristeromycin and Neplanocin A

2. EXPERIMENTAL METHODS

2.1 Fermentations, HPLC and FTIR analysis

Streptomyces citricolor strains were grown in GAM medium (glucose 60g; Arkasoy 50, 60g; MOPS, 21g; per litre water, pH7.0) in duplicate 250ml fermentation flasks at 28°C. Samples extracted after 3 and 6 days incubation were centrifuged and the supernatant diluted 1:20 in a 1:1 mixture of methanol and mobile phase (20% acetonitrile containing 3g/l SDS, 0.25mM $NH_4H_2PO_4$ and 0.5ml H_3PO_4 per litre). After centrifugation the clarified supernatants were assayed by reverse phase HPLC on a C18 Spherisorb 5μ 150x4.6mm column at a flow rate of 2ml min^{-1}. Aristeromycin and neplanocin A were quantified from relevant peak areas representing absorbance at 260nm after calibration with standards of known concentration. HPLC equipment consisted of a Varian Star 9010 solvent delivery system and 9050 variable wavelength UV-VIS detector.

DRIFT analysis was performed using a Bruker IFS28 infra-red spectrometer equipped with a diffuse-reflectance TLC attachment (Bruker Spectrospin Ltd., Banner Lane, Coventry CV4 9GH, U.K.) and a liquid N_2-cooled MCT (Mercury-Cadmium-Telluride) detector. Unprocessed culture supernatants (5μl; four replicates) were dried in the wells of a sand-blasted aluminium plate mounted on a motorised stage and infra-red spectra were collected in the range 4000/cm^{-1}-600/cm^{-1} with 1 or 16 co-adds. Spectral data were converted into ASCII format for chemometric processing.

3. RESULTS AND DISCUSSION

3.1 Multivariate Analysis

The similarity between the diffuse reflectance FTIR spectra of aristeromycin and neplanocin A (Figure 2) and the complexity of the supernatant background FTIR spectra (see Figure 3) necessitates the use of multivariate methods for quantitative analysis of the components. Training data for the construction of multivariate calibration models and test data for model validation were provided by HPLC and FTIR analysis of the *S. citricolor* mutant fermentation supernatants with a total of 48 samples taken from duplicate fermentations after 3 and 6 days incubation.

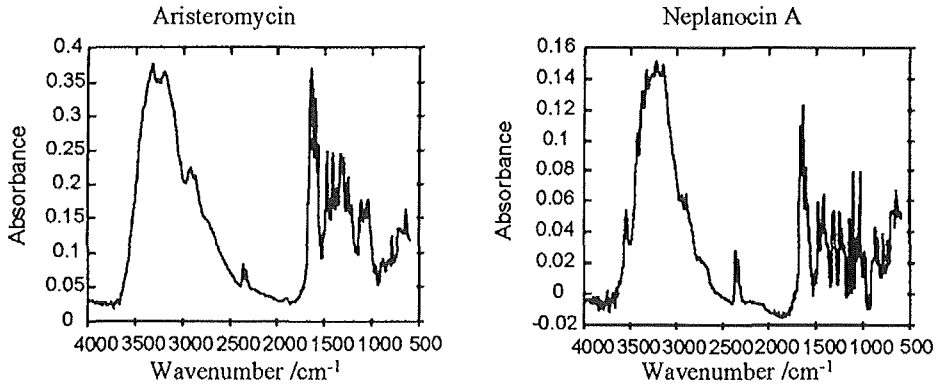

Figure 2. Representative FTIR absorbance spectra of Aristeromycin and Neplanocin A.

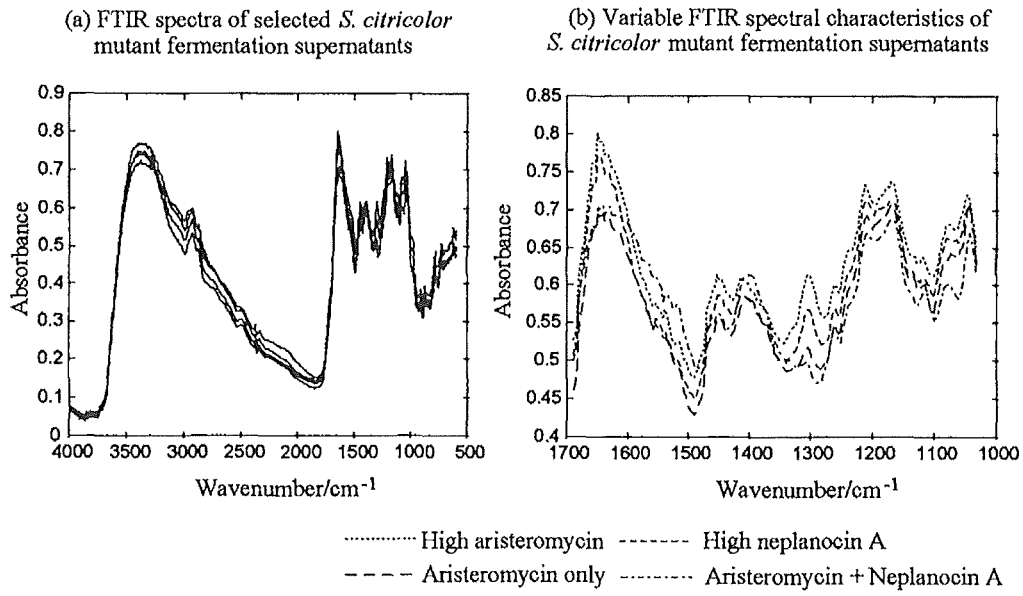

Figure 3. FTIR absorbance spectra of supernatants from four different *S. citricolor* mutants in the wavenumber ranges **(a)** 4000-600/cm-1; **(b)** 1700-1000/cm-1. The background variation of the *S. citricolor* mutant culture supernatants and the general similarity between the FTIR spectra of aristeromycin and neplanocin A (see Figure 2) necessitates the use of multivariate data analysis for quantification and classification.

To ensure that the training data for input into multivariate calibration methods encompassed the full range under study a variation of the Duplex method [6] called "Multiplex" [7] was used to split the data. Multiplex-processed data were then used as inputs for multivariate analysis.

PLS (Partial Least Squares) regression was used for quantification and classification of aristeromycin and neplanocin A (Figure 4). Matlab was used for PCA (Principal Components Analysis) (according to the NIPALS algorithm) to identify correlations amongst the variables from the 882 wavenumbers and reduce the number of inputs for Discriminant Function Analysis (DFA) (first 15 PCA scores used) (Figure 5).

In analysing complex fermentor broths and supernatants, in contrast to the case for simple mixtures, the multivariate analysis approach not only monitors the infra-red spectral contribution of the target molecules *per se* but may also take into account other features associated with the metabolite concentration. In this way the use of unsupervised methods such as PCA and supervised methods such as PLS, and DFA can exploit differences in the organism (for example the mutant type) or the medium which correlate with metabolite overproduction. This can provide a useful 'amplification' to the method when the target molecule concentrations are particularly low.

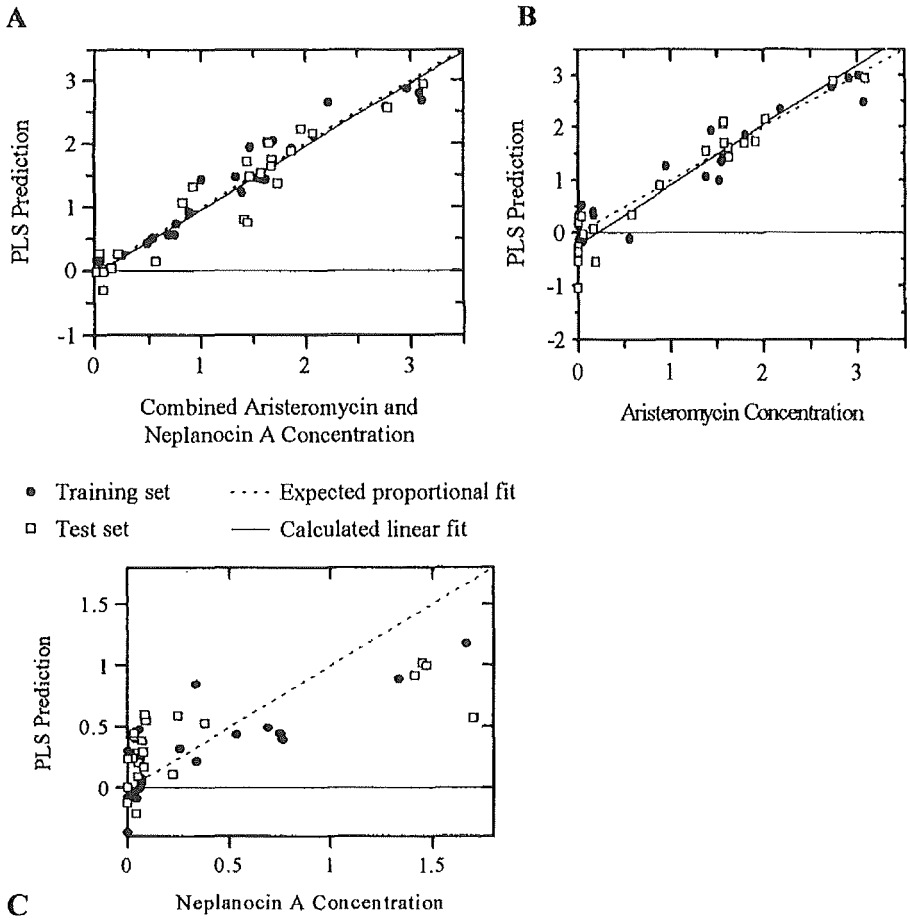

Figure 4. PLS predictions of **(A)** combined aristeromycin and neplanocin A concentration (6 factors, MSEP 0.088); **(B)** aristeromycin concentration (7 factors, MSEP 0.1391); **(C)** neplanocin A concentration (5 factors, MSEP 0.141) from all of the *S. citricolor* mutant fermentation supernatants (g. l^{-1}). Circles represent samples in the training set used to form the PLS model and squares indicate the predicted concentrations derived from 'unknown' spectra that were not in the training set. Aristeromycin and neplanocin A concentrations were obtained by HPLC analysis.

3.2 Classification

The rapid screening for metabolite overproduction facilitated by the DRASTIC method means that a substantially higher throughput of candidate mutants can be achieved in a given time. We envisage that this increased coverage should permit detection of rare mutants which overproduce to a level only encountered infrequently (or not at all) during strain development programmes. In this scenario it is sufficient to determine overproduction of a particular metabolite empirically by assigning a class where this criterion is satisfied.

A classification model based on assignment to categories of high or low production was used to distinguish between four classes of mutants, including two classes overproducing aristeromycin or neplanocin A. A threshold value of 0.1g l^{-1} for both molecules was used to define high or low producers for classification by DFA (Figure 5.) and PLS (data not shown).

3.3 Data interpretation

Chemical mutagenesis of microbial strains typically produces mutants with a range of metabolite overproduction capabilities. In the case of *S. citricolor*, mutants have been isolated which produce either aristeromycin, or neplanocin A, or both molecules, at higher levels. In strain development programmes efficient identification of those mutants overproducing target molecules is of key importance.

Although in many instances it might be possible to identify and quantify different molecules of interest simultaneously, in other cases accurate quantification by FTIR analysis without further sample processing may be complicated by the nature of the molecules under investigation. The use of multivariate spectral information is advantageous where quantification of a particular metabolite in a complex biological background is being attempted; however, *accurate* quantification in the presence of another component with a similar FTIR spectrum may be dependent upon very few unique spectral features. Under these circumstances it may be necessary to determine the presence or absence of the molecule of interest at a threshold level in order to permit further analysis by other chemometric or wet analytical methods.

Quantification of neplanocin A in the presence of aristeromycin was found to be such an example. Accurate quantification of neplanocin A under these circumstances was not possible using PLS regression performed on the whole data set (see Figure. 4); however, PCA followed by DFA was successfully used to discriminate between the mutant classes (Figure 5). Where sufficient representative samples are available quantitative analysis could then be performed on these subclasses alone.

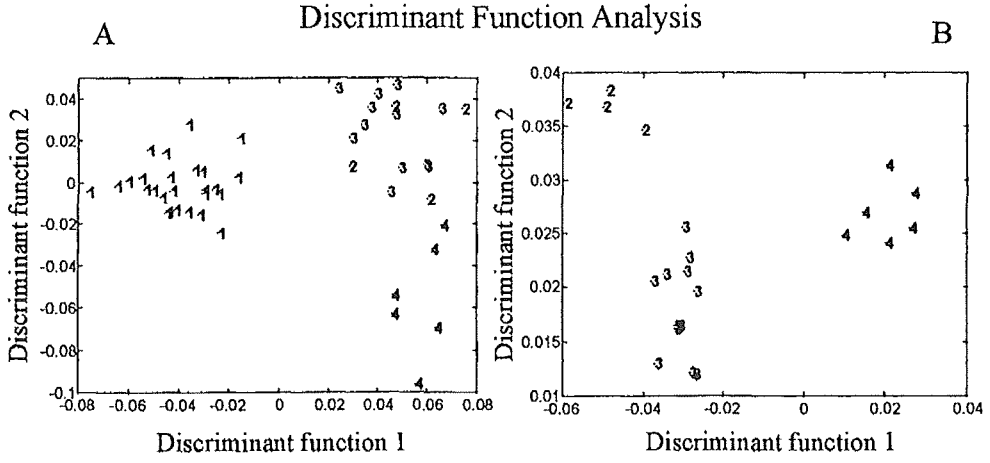

Figure 5. Separation of *S. citricolor* mutant classes by DFA; Twelve different strains were assigned to four classes on the basis of their antibiotic production; **1)** high aristeromycin; **2)** both aristeromycin and neplanocin A; **3)** high neplanocin A; **4)** low aristeromycin and low neplanocin A. **(A)** Using all 4 classes as input; **(B)** using 3 classes as input after discrimination and removal of the class 1 cluster.

4. CONCLUSIONS

The results of this study, using quantification of aristeromycin and neplanocin A in a model *S. citricolor* fermentation system, illustrate for the first time that the DRASTIC approach provides a novel, rapid and successful method for screening microbial strains for metabolite overproduction in titre improvement programmes.

5. ACKNOWLEDGEMENTS

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