Measurement of mycelial biomass in a submerged bioprocess using near infrared spectroscopy

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Introduction

Biomass is a key analyte, the estimation of which is an important exercise in bioprocess monitoring.[1] Several techniques have been investigated over the years for the measurement of this analyte. Among these, optical techniques and, in particular, NIR has shown good potential for on-line measurement.[2–5] Although biomass measurement using NIR has been demonstrated for bioprocesses employing unicellular micro-organisms,[4–6] very little has been reported on the application of the technique for monitoring mycelial biomass. Bioprocesses employing mycelial biomass comprise a major group of commercial importance, especially in the pharmaceutical industry. Applying optical techniques for monitoring mycelial biomass is challenging due to the morphological variation that mycelial biomass displays within and between process runs, due to the filamentous nature of the micro-organism comprising the biomass. A submerged bioprocess involving *Penicillium chrysogenum*, a fungus, was investigated. *P. chrysogenum* was chosen as it is a typical mycelial micro-organism that produces a commercially important bulk antibiotic, penicillin. We present here the feasibility of monitoring mycelial biomass using NIR and discuss the evaluation of multivariate calibration equations that were developed for the measurement.

Materials and methods

Bioprocess

P. chrysogenum (an industrial strain supplied by SmithKline Beecham Pharmaceuticals. Irvine, UK) was cultivated in a 10 L Biostat ED ES10 bioreactor (B. Braun Biotech International, Melfungen, Germany). Fungal spores developed on rice were used to prepare an inoculum in a medium containing (in g L^{-1}) lactose—70, sucrose—10, $(NH_4)_2SO_4$ —7.0, K_2HPO_4—1.6, $FeSO_4.7H_2O$—0.04, $MgSO_4.7H_2O$—0.1, KCl—0.5, $CaCl_2$ —0.04, $MnSO_4.H_2O$—0.02, $ZnSO_4.7H_2O$—0.02 and $CuSO_4.5H_2O$—0.005, pH—5.9. A 10% (v / v) inoculum was used to inoculate 10 L of the same medium in the bioreactor. The culture was grown at 25°C, at a pH of 5.9, with an agitation of 800 rpm and an aeration rate of 1 vvm.

Samples were withdrawn at regular intervals and their NIR spectra acquired. Simultaneously, they were assayed for biomass, by a reference assay.

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Reference assay

Biomass was measured by filtering a weighed aliquot of the broth sample through a preweighed Whatman GF/C filter paper. The residue was dried in a microwave oven (600 W) at low power to constant weight and the dry cell weight estimated by gravimetric difference.

NIRS measurements

The spectra were acquired with a Model 6500 NIR spectrophotometer (Foss-NIRSystems, Silver Spring, MD, USA) in the transmittance mode with a cuvette of 1 mm pathlength, using the sample transport module. 32 co-added scans of the samples were referenced with 32 co-added scans of air as reference. Samples were scanned in triplicate. The raw spectra were derivatised and the second order derivatives used throughout the investigation. A segment size of ten and a gap size of two was used for the derivatisation.

Model development

The data set was divided into calibration and validation sets. Samples from three independent bioprocess runs were used to build the calibration models, while a fourth run was used to validate the models developed. Four different calibration and validation sets were generated by a combination of the set of four runs (Bio. 1–4). The statistical techniques, multiple linear regression (MLR) and partial least squares (PLS), were used to develop the models. The software NSAS (Foss-NIRSystems, Silver Spring, MD, USA) was used throughout the exercise.

External validation

Samples were obtained from two different runs. The first run, Bio 5, comprised of samples from a *P. chrysogenum* bioprocess run, carried out under the same conditions as those used in developing the models. The samples were centrifuged (8,000 rpm, 4°C, 20 min.) to harvest the biomass which was then resuspended back in the supernatant to generate samples with different biomass concentration (Bio 5sp). Manipulation of the sample in this manner introduces variation in the biomass concentration, but keeps matrix changes to a minimum. The second run, Bio 6, comprised a *P. chrysogenum* bioprocess run, carried out under the same conditions as Bio 5, except that it was run in a 2 L bioreactor (Setric Genie Internationale, Toulouse, France), with a single stirrer. The difference in the bioreactor environment resulting from a reduction in the scale of operation might reasonably be expected to introduce changes in biomass morphology, apart from influencing physiological parameters, such as biomass concentration, substrate uptake and product consumption. Samples from this run were thus intended to challenge the robustness of the models, under different process operational conditions.

Results and discussion

The models are evaluated and their characteristics are summarised in Table 1. For the MLR models, three wavelength terms were used. The primary wavelength term in all cases can be traced to the contribution from biomass.[7] Absorbances at the other two wavelengths were found to have contributions from the culture medium (sugars, ammonium at the early stage of the bioprocess and penicillin at the later stages). For the PLS models, a factor size of 4 was found to be optimal (Figure 1) for all the calibration set combinations.

On external validation, samples from a run carried out under conditions identical to that employed for model development, perform well (Bio 5), as can be seen from the standard error of prediction (*SEP*) and *r* values in Table 2. An inspection of the correlation plots (Figures 2 and 3) shows that the points are evenly distributed about the 45° line, with a tight fit, suggesting a good agreement between

Table 1. Models evaluated and their characteristics. Bio 1,2,3,4 are replicate runs of the *P. chrysogenum* bioprocess; MLR - multiple linear regression; PLS—partial least square; *SEC*—standard error of calibration,; R^2—coefficient of multiple determination.

Model	Calibration set	No. of samples	Concentration range ($g\,l^{-1}$)	*SEC*	R^2	Validation set	No. of samples	Concentration range ($g\,l^{-1}$)
MLR 1—1724, 1644, 1684 nm PLS 1—1600 – 1800 nm, f=4	Bio. 1,2,3	76	0.9 – 21.4	0.903 0.720	0.969 0.980	Bio. 4	25	1.7–18.1
MLR 2—1724, 1646, 1686 nm PLS 2 —1600– 1800 nm, f=4	Bio. 2,3,4	77	0.9 – 18.1	0.988 0.740	0.964 0.978	Bio. 1	24	1.7–18
MLR 3—1724, 1638, 1682 nm PLS 3— 1600– 1800 nm, f=4	Bio. 3,4,1	76	1.7 –21.4	1.060 0.620	0.954 0.988	Bio. 2	25	2.3–21.4
MLR 4 —1722, 1632, 1682 nm PLS 4— 1600–1800 nm, f=4	Bio. 2,4,1	74	0.9 – 21.4	0.820 0.699	0.979 0.982	Bio. 3	27	0.9–13.3

the two data sets, for the models evaluated on Bio 5. The same is observed for the manipulated samples (Bio 5sp), although the scatter tends to have a slight bias for the PLS models, at higher biomass concentration. Since the sample manipulation method adopted changes only the biomass concentration, keeping matrix changes to a minimum, the performance of the models indicates that, in all likelihood, biomass and not any other component of the sample matrix is being modelled. It is interesting to note that despite morphological heterogeneity expected in a given sample and within a process run, the models perform well for all the calibration set combinations.

Table 2. Performance of the models on external validation. Table lists the *SEP* and *r* values for the three validation sets Bio 5, Bio 5sp and Bio 6.

Model	Bio. 5 $n = 7$	Bio. 5sp $n = 1$	Bio. 6 $n = 10$
MLR1			
SEP	0.895	0.882	1.860
r	0.987	0.985	0.839
MLR2			
SEP	0.893	0.842	1.560
r	0.987	0.986	0.890
MLR3			
SEP	1.060	1.040	2.020
r	0.981	0.979	0.808
MLR4			
SEP	1.230	1.140	1.750
r	0.974	0.974	0.859
PLS1			
SEP	0.617	0.754	1.250
r	0.994	0.989	0.931
PLS2			
SEP	0.946	0.834	1.680
r	0.985	0.986	0.871
PLS3			
SEP	0.606	0.762	1.200
r	0.994	0.989	0.937
PLS4			
SEP	0.900	1.540	0.814
r	0.986	0.953	0.971

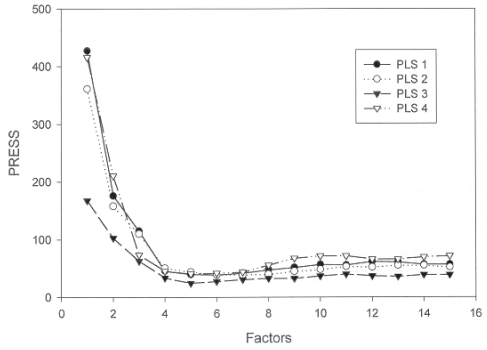

Figure 1. Change in PRESS values with increase in the number of factors used in the development of the PLS models. Note the uniformity in behaviour for all the four combination sets. An optimum factor size of 4 can be inferred, for all the combinations.

However, evaluation of the models on a bioprocess that was run on a different scale (Bio 6), shows higher *SEP* values and weaker correlation. Both with the MLR and the PLS models, the correlation seems to have a strong bias for most of the calibration set combinations. It is possible that morphological variations in the mycelial biomass, resulting from a change in the scale of operation, influence the NIR measurements. This is a subject of further investigation.

Conclusions

This work demonstrates the feasibility of measuring mycelial biomass, using NIR. The models show good predictive capabilities when tested on samples obtained from a run operated under conditions similar to that used for model development, despite the morphological varia-

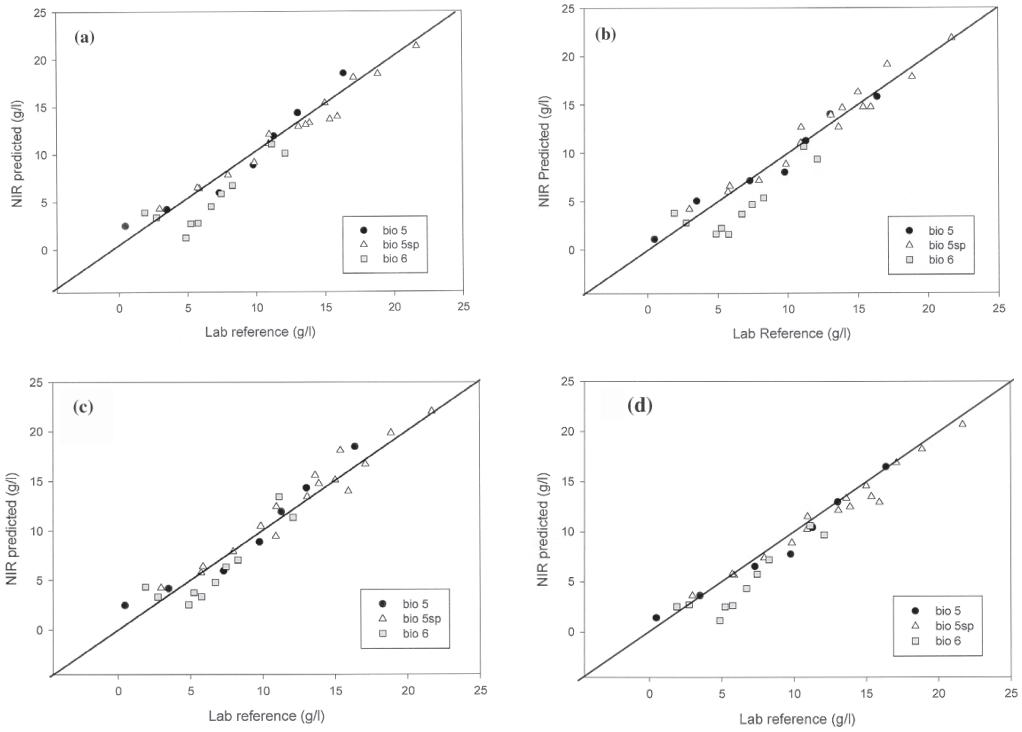

Figure 2. Correlation plots of the NIRS predicted and reference assay data for the MLR models (a) MLR1; (b) MLR2; (c) MLR3; and (d) MLR4.

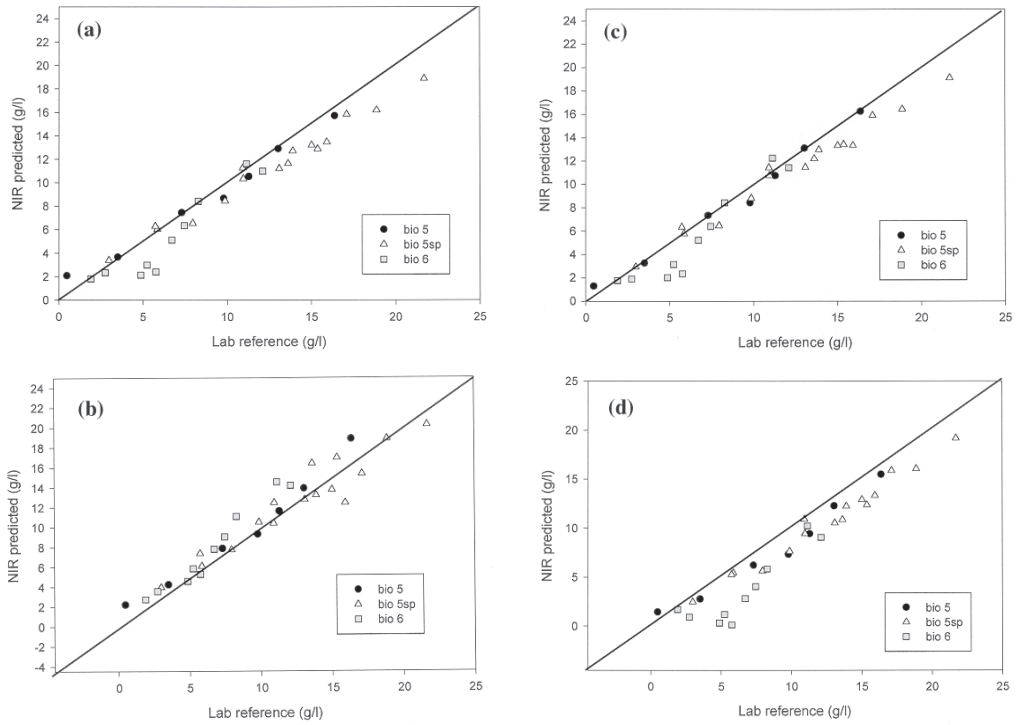

Figure 3. Correlation plots of the NIR predicted and reference assay data for the PLS models (a) PLS1, (b) PLS2, (c) PLS3 and (d) PLS4.

tions expected within the process. However, they do not perform as well when tested on samples from a process operated on a different scale. Morphological and physiological variations may influence the measurements in this context.

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