



Focussed beam reflectance measurement (FBRM) monitoring of particle size and morphology in suspension cultures of *Morinda citrifolia* and *Centaurea calcitrapa*

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Abstract

Laser light scattering technology, as applied in the Lasentec focussed beam reflectance measurement (FBRM) system, was used to characterise two morphologically dissimilar plant cell suspension cultures, *Morinda citrifolia* and *Centaurea calcitrapa*. Shake-flask suspensions were analysed in terms of biomass concentration and aggregate size/shape over the course of typical batch growth cycles. For the heavily aggregated *C. calcitrapa*, biomass levels [from 10–160 g fresh weight (fw) l⁻¹] were linearly correlated with FBRM counts. For *M. citrifolia*, which grows in unbranched chains of 2–10 elongated cells, linear correlation of biomass concentration with FBRM counts was applicable in the range 0–100 g fw l⁻¹; at higher levels (100–300 g fw l⁻¹), biomass was non-linearly correlated with FBRM counts and length-weighted average FBRM chord length. For both cell systems, particle morphology (size/shape) was quantified using semi-automated digital image analysis. The average aggregate equivalent diameter (*C. calcitrapa*) and average chain length (*M. citrifolia*), determined using image analysis, closely tracked the FBRM average chord length. The data clearly demonstrate the potential for applying the FBRM technique for rapid characterisation of plant cell suspension cultures.

Introduction

Despite their significant biochemical potential, industrial-scale application of plant cell suspension cultures is limited, primarily due to a combination of economic and engineering factors (Kieran 2001). Biomass levels [up to about 20 g dry wt (dw) l⁻¹, equivalent to approx. 300–400 g fw l⁻¹] are higher than those typically achieved in bacterial systems and the suspensions are generally heavily aggregated. Due to the characteristically longer doubling times of plant cells (approx. 20–100 h) batch duration is extended and system sterility is challenged. Under these circumstances, it may be difficult to obtain a representative broth sample from a bioreactor; moreover, sampling

may increase the risk of contamination. The availability of robust, *in situ* methods for evaluation of key process variables would obviate the need for sampling.

Focused beam reflectance measurement (FBRM) technology, implemented in a probe which may be installed directly in a reactor, has been successfully used for the *in situ* characterisation of particle size and concentration in chemical processing applications (e.g. Barrett & Glennon 1999, 2002, Loan *et al.* 2002). As the probe may be exposed to sterilisation temperatures, it is also suitable for bioreaction applications. To date, the FBRM system has been employed for biomass measurement and morphological characterisation in filamentous systems (Pearson *et al.* 2003). Working with three different suspensions

(*Oryza sativa*, *Nicotiana benthamiana* and *Trichosanthes kirilowii*), McDonald *et al.* (2001) demonstrated the feasibility of applying this technique to plant cell systems. Briefly, the probe operates by projecting a highly focused laser beam, through a set of rotating optics and a sapphire window, into a suspension sample. The beam traces a path around the window circumference. When a particle intersects the beam, the laser light is backscattered. This backscatter is measured: the duration of the backscatter is related to the size of the particle and is expressed in terms of a chord length; the number of such chords measured from a given suspension sample, under specified measurement conditions and in a specified time period, is related to the particle concentration. Comprehensive details on the operation of the FBRM system are presented elsewhere (Barrett 2002).

Methods

Cell cultures

Cultures of *Morinda citrifolia* and *Centaurea calcitrapa* were grown in 250 ml and 1 l shake-flasks, respectively (with working volumes of 100 ml and 500 ml, respectively). All flasks were maintained on an orbital incubator (120 rpm) at 25 °C. *M. citrifolia* was subcultured every 14 d, as described elsewhere (Zenk *et al.* 1975). *C. calcitrapa* was subcultured weekly, using a 20% (v/v) inoculum and a modified Schenk and Hildebrandt medium, supplemented with 30 g l⁻¹ glucose, 1.5 mg l⁻¹ naphthaleneacetic acid and 0.15 mg l⁻¹ kinetin; *C. calcitrapa* flasks were kept in the dark. Full cultivation details are provided by Raposo (2003).

FBRM measurements

Using an FBRM probe (Model M400LF, Lasentec Inc., Redmond, WA), all suspension samples were analysed in the same manner. In a 400 ml beaker, 160 ml cell suspension (either neat or diluted) was stirred at 200 rpm, using a pitched-blade, upward-pumping impeller (diam. 50 mm). The FBRM probe, mounted in a proprietary Lasentec stand, was submerged in the suspension to a depth of 2.5 cm; the window of the probe faced downward into the direction of flow. Twenty measurements, each of 10 s duration, were made on each sample, and the data recorded using the FBRM acquisition software. The data were binned into 90 logarithmically-spaced channels,

in the range 1–1000 µm. Data were exported to Excel for further manipulation. Where indicated, neat cell suspensions were diluted with KNO₃ at 4 g l⁻¹. As FBRM results are susceptible to the choice of the focal point position (Worlitschek & Mazzotti 2003), for comparison between *M. citrifolia* and *C. calcitrapa*, count data are presented on relative bases.

Morphological analysis

Because the FBRM system measures particle chord length, rather than a specific dimension (e.g. diameter or maximum feret), results are sensitive to both particle shape and particle size. Accordingly, two morphologically dissimilar cell suspensions were selected for this study. In suspension culture, *C. calcitrapa* is heavily aggregated, with cells forming roughly spherical aggregates, of 50–3000 µm equivalent diameter (Raposo 2003). This aggregation is characteristic of many plant cell systems. In contrast, *M. citrifolia* cells are characteristically cylindrical in shape and exist in unbranched chains of between 2 and 10 cells. Chains are typically between 100 and 1700 µm in length and approx. 25–50 µm in width (Kieran *et al.* 1993). This morphology is less common in plant cell cultures, although Curtis & Emery (1993) reported similar characteristics for suspensions of *Nicotiana tabacum* cultivated under batch conditions.

For morphological measurements, suspension samples, stained with Evan's Blue for improved contrast, and appropriately diluted with KNO₃ at 4 g l⁻¹, were loaded into a custom-built glass chamber (1.5 mm in depth) mounted on a microscope slide and covered with a coverslip. The dilution factor was adjusted, depending on the original biomass concentration, to reduce the incidence of aggregate overlap, when viewed under the microscope. Semi-automated image analysis was performed using a Leica Q500IW image analysis system with QWIN software version 2.3, coupled to an Olympus BX-2 microscope. Samples were viewed under a 4× Dplan lens. Analysis was performed using a self-authored macro written using the QUIPS macro programming functions. *C. calcitrapa* samples were analysed on the basis of equivalent aggregate diameter; for *M. citrifolia*, the area and perimeter of each chain was determined. For statistical robustness, at least 300 entities were analysed to produce the distributions presented here. This minimum was identified on the basis of a 'running average' as described by Pearson *et al.* (2003). The data were binned into geometrically spaced bins (80–

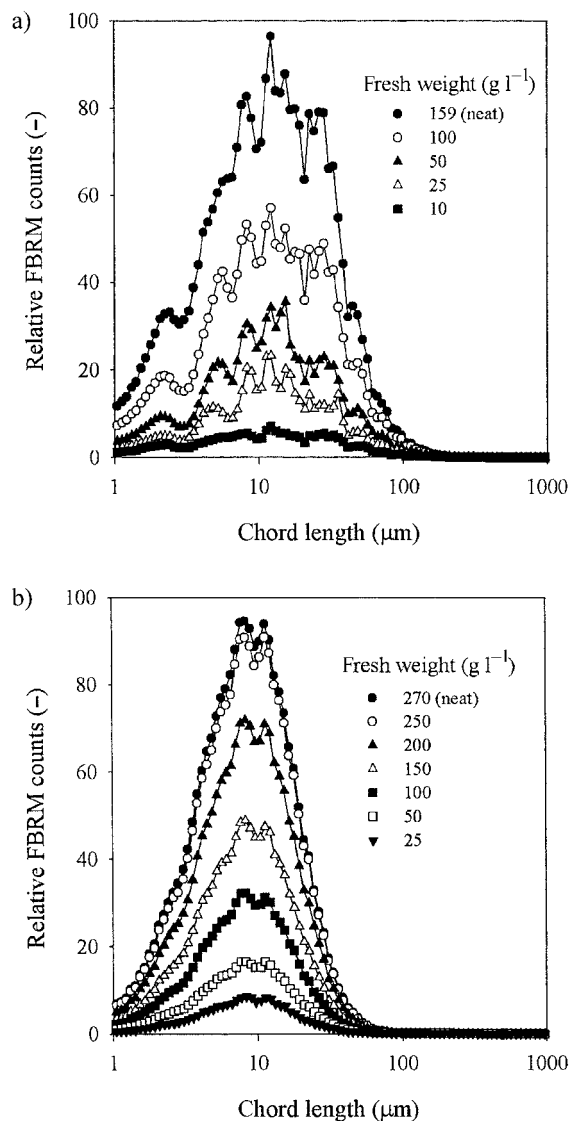


Fig. 1. FBRM chord length distributions for (a) *M. citrifolia* and (b) *C. calcitrapa* suspensions, neat and progressively diluted with a 4 g l^{-1} aqueous solution of KNO_3 . FBRM count data in these figures have been scaled to account for variations in the FBRM focal point employed for the two cell systems.

$2551 \mu\text{m}$) to which log-normal distributions were then fitted using non-linear regression techniques.

Results and discussion

Effect of biomass level on FBRM chord length distribution

In batch culture, plant cell suspensions typically achieve significantly higher biomass levels than bac-

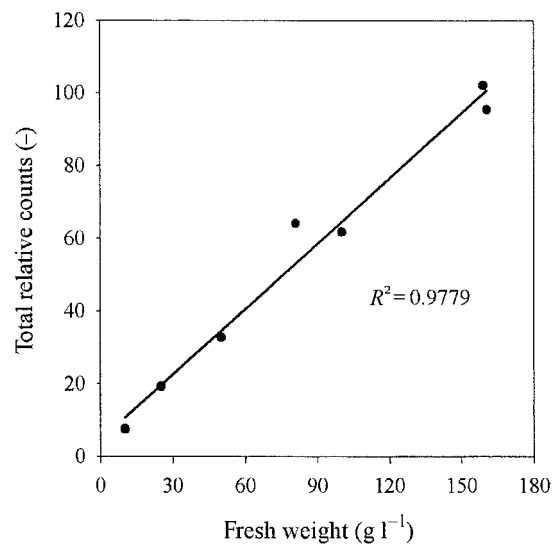


Fig. 2. Relationship between total FBRM counts and fresh cell weight for *C. calcitrapa*.

terial and mammalian cell systems. In this laboratory, *M. citrifolia* and *C. calcitrapa* achieve maximum biomass (fresh weight) levels of approx. 300 g l^{-1} and 200 g l^{-1} , with corresponding dry weights of approx. 14 g l^{-1} and 10 g l^{-1} , respectively. However, values of the fw/dw ratio, indicative of higher cellular water content and larger cell sizes than in bacterial systems, are both line-specific and dependent on cultivation conditions. For this study, fresh weight values – which more directly reflect the condition of the biomass in suspension – were employed as the correlating parameter, in preference to dry weight levels. For both suspensions, the ability of the FBRM to detect changes in biomass levels was successfully demonstrated using samples of diluted broth. Figure 1 shows (unnormalised) chord length distributions for broths of (a) day 7 *C. calcitrapa* and (b) day 10 *M. citrifolia* broths, progressively diluted from original biomass of 159 g l^{-1} and 270 g l^{-1} to 10 g l^{-1} and 25 g l^{-1} , respectively. In both cases, total FBRM counts increase with increasing cell concentration. Although the shape of the FBRM count distributions appears to be system specific, for each culture, internal consistency is confirmed by the insensitivity of the shape of the distribution to sample dilution.

Figure 2 shows a strong linear relationship between total FBRM counts and *C. calcitrapa* biomass (fresh weight) levels over the course of a 7-d batch growth cycle, clearly demonstrating the potential of the FBRM to track changes in biomass concentration in an

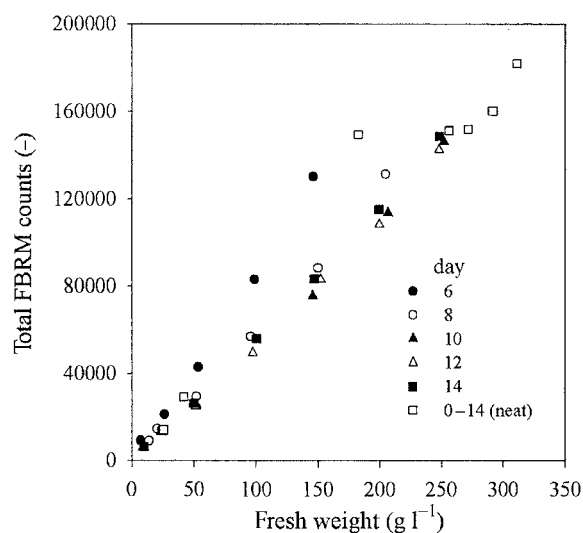


Fig. 3. Relationship between total FBRM counts and fresh cell weight for *M. citrifolia*.

aggregated suspension. The relationship between biomass concentration and FBRM counts for *M. citrifolia* is more complex. Figure 3 shows the variation in total FBRM counts with fresh weight for neat (whole broth) and diluted suspensions, harvested across the 14-d growth cycle. (For clarity, data for samples harvested on days 0, 2 and 4 have been omitted.) Here again, as with *C. calcitrapa*, there is a strong, positive correlation between total counts and fresh weight. However, closer examination of the data reveals deviations in the day 6 data, particularly at higher biomass levels. These deviations are explained in terms of size-related effects (see Influence of particle size on FBRM-based biomass correlation).

Changes in particle size

As a size reference for FBRM measurements, McDonald *et al.* (2001) used sieving, which proved less suitable than image analysis for both the highly friable *C. calcitrapa* suspensions and the heavily elongated *M. citrifolia* chains. Furthermore, image analysis of wet samples permits characterisation under conditions more representative of actual processing conditions than sieving.

To investigate the possibility of tracking changes in *C. calcitrapa* aggregate size, two FBRM-based statistics were studied: the mean chord length and the mean square-weighted chord length. These values were compared to the average aggregate equivalent diameter, determined using image analysis. As

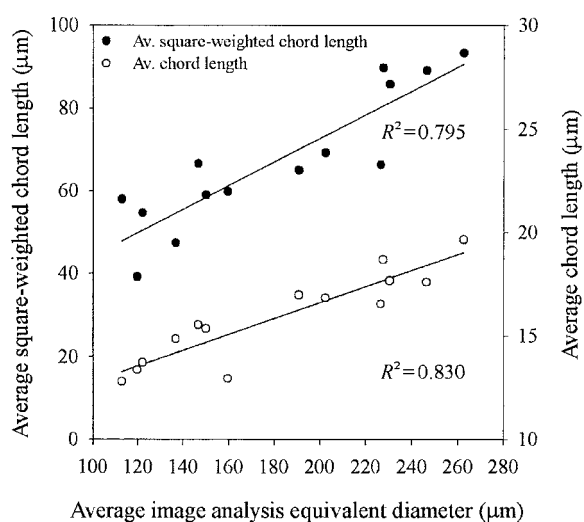


Fig. 4. Correlation between *C. calcitrapa* average aggregate equivalent diameter (determined using image analysis) and two FBRM-based statistics, the average chord length and the average square weighted chord length.

measured by the FBRM, chord length is not just a function of particle size, but is also determined by particle shape, opaqueness and surface properties. Nonetheless, Figure 4 shows a strong linear correlation between average aggregate equivalent diameter and both FBRM-based statistics, with average chord length yielding a slightly higher correlation coefficient ($R^2 = 0.8303$).

Figure 5a shows normalised FBRM chord length distributions for the first 8 d of the *M. citrifolia* growth cycle. Between days 0 and 2, there is a pronounced shift to the right as well as an increase in the spread of the distributions, reflecting increasing particle (chain) size, with little further change at day 6. The day 8 chord length distribution is very similar to that recorded on day 0 and there are no subsequent variations throughout the remainder of the batch cycle (data not shown). These patterns are generally mirrored by the corresponding image analysis data (Figure 5b), to which log-normal distributions have been fitted. The shift in the distributions to the right indicates an increase in cell length during the lag and exponential growth phases, with an associated increase in chain length. Thereafter, larger chains sub-divide, resulting in a shift in the distribution to the left. After day 8 of the 14-d growth cycle, there is little variation in any morphological characteristic. Good agreement between the trends in the time profiles for both average chain length (image analysis) and average FBRM chord length (Figure 6) shows the potential for using

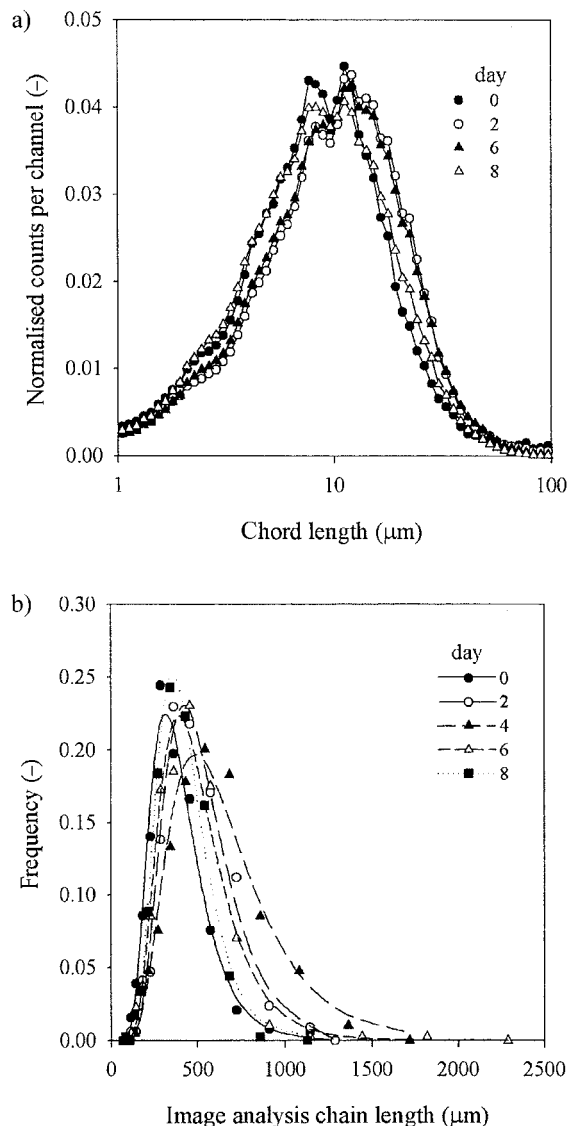


Fig. 5. (a) Normalised FBRM chord length distributions and (b) image-analysis based frequency distributions for chain length for *M. citrifolia*. The log-normal curves fitted to the experimental data in (b) were obtained using the Solver function on Microsoft Excel.

the FBRM, as an alternative to image analysis, for tracking changes in particle size.

Influence of particle size on FBRM-based biomass correlation

During the lag and early growth phases, the image analysis data presented in Figure 5b reveal that the average *M. citrifolia* chain length increases by approx. 43%. By contrast, in the *C. calcitrapa* suspensions, the average aggregate equivalent diameter varies by

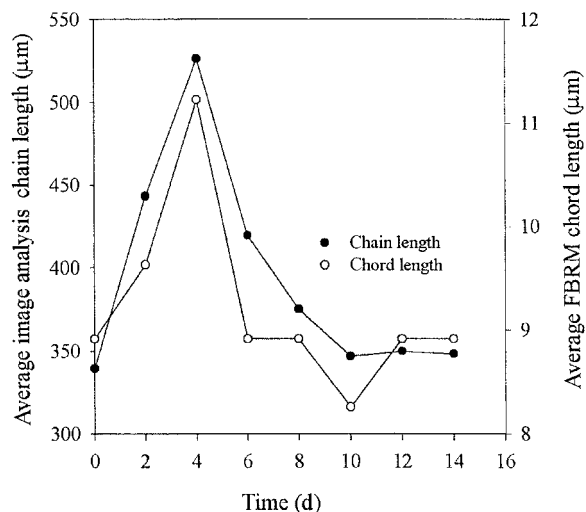


Fig. 6. Variation of both the average image analysis chain length and the average FBRM chord length for *M. citrifolia* over the course of a 14-d batch growth cycle.

only 29% during the week-long batch cycle (data not shown). As the FBRM chord length distributions (Figure 2) reflect particle number, size and geometry, a simple correlation of FBRM counts with biomass concentration may be less appropriate in the context of significant changes in particle size distributions. For *M. citrifolia*, non-linear regression yields the following empirical relationship between cell fresh weight (FW), total FBRM counts recorded over a 200 s measurement period (N) and the length-weighted mean chord length, Y_l :

$$FW = 530 N^{0.45} Y_l^{-2.1},$$

where Y_l is calculated from the number of counts, n_i , recorded in a bin of midpoint L_i , as

$$Y_l = \frac{\sum_{i=1}^{90} n_i L_i^2}{\sum_{i=1}^{90} n_i L_i}.$$

In Figure 7, this correlation is applied to two independent sets of *M. citrifolia* batch data, including measurements for neat and diluted samples, at biomass levels in excess of 100 g l^{-1} ; at lower biomass levels, a simple linear relationship is applied. There is very good agreement between experimental and predicted values.

The results presented here clearly illustrate the potential of the FBRM probe for monitoring morphologically diverse systems, at biomass levels as high as 250 g fw l^{-1} (approx. 14 g dw l^{-1}). FBRM-based correlations for biomass levels and aggregate size developed for both *M. citrifolia* and *C. calcitrapa* proved

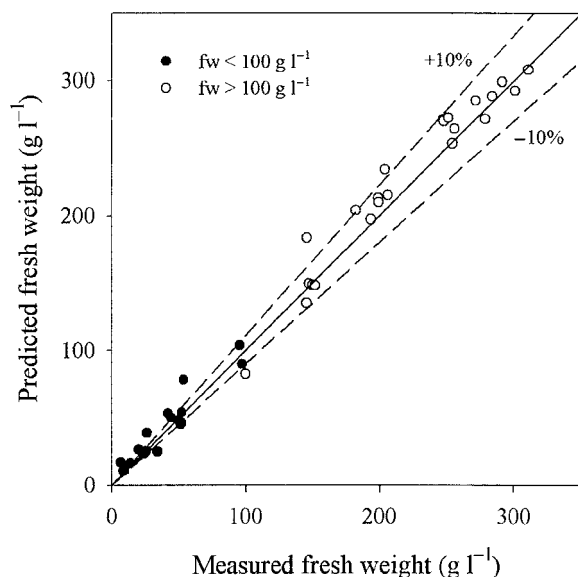


Fig. 7. Predicted and experimentally-determined biomass values for *M. citrifolia*. For biomass levels in excess of 100 g l^{-1} , the non-linear correlation is based on FBRM counts and length-weighted average chord length; below 100 g l^{-1} , biomass levels are linearly correlated with FBRM counts. The dashed lines represent $\pm 10\%$ of the experimental values. Data presented were collected from two independent batch cultures and include both whole broths and diluted samples.

more successful for these systems than correlations based on the cube-weighted FBRM chord length, proposed by McDonald *et al.* (2001) for rice, tobacco and wild Chinese cucumber, reflecting the system-specific nature of the technique.

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