Quantification of cell size distribution as applied to the growth of *Corynebacterium glutamicum*

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**KEYWORDS**

Cell size distribution; Image analysis; Cumulative distribution function; Growth characteristic; *Corynebacterium glutamicum*

**Summary**

It is known that the cell size is related to the physiological state of a cell. Therefore, cell size distribution directly reflects the average physiological properties of the cell culture. Cell size distribution can be enumerated by image analysis, flow cytometry and coulter counter. In this study, image analysis was used to characterize the cell size distribution during the growth of *Corynebacterium glutamicum* and was further analyzed by a distribution function. The parameters of the distribution function indicate the mean value and spread of the distribution. Analysis demonstrated that the maximum specific growth rate was higher (0.67 h\textsuperscript{-1}) for the growth obtained through serial dilution of seed as compared to growth from a normal seed culture (0.53 h\textsuperscript{-1}). This was due to a greater percentage of the cell population being in the state of division for the growth through serial dilution in the mid-log phase. The measurement of the cell size distribution demonstrated that the average cell size decreased during the course of growth. The distribution function was also used to enumerate the average specific growth rate of both the conditions of the culture. The demonstrated methodology can be used to predict an average growth property of a cell culture.

**Introduction**

Performance of a biotechnological process depends on the physiological state of the growing biomass. The physiological state, which may be affected by the media composition, can be directly related to the cell population. Most studies typically look at average measurements of cell property and very little information is available on the distribution of cell population (Porrot and Friedrich, 1995). Therefore, study of cell size distribution is essential to gather precise knowledge about the state of the organism. Several methods, such as coulter counter, flow cytometry and image analysis, have been reported for cell size
measurement (Boyea and Olesen, 1991; Neagu et al., 1994; Porrot and Friedrich, 1995; Porrot et al., 1997).

Among these, image analysis is a very promising and powerful tool to quantify cell distribution based on size. The method offers advantage to directly observe individual cells, cell aggregates or differentiated cell masses (Ibaraki and Kenji, 2001). Image analysis has been applied to evaluate microorganisms in various environments including pure and mixed cultures (Pons and Vivier, 2000), marine (Pernthaler et al., 2003), petrochemicals (Williamson and Palframan, 1989), geological (Toebot et al., 1999) and foods (Hood and Zottola, 1997). Image analysis has been applied to evaluate microorganisms in various environments including pure and mixed cultures (Pons and Vivier, 2000), marine (Pernthaler et al., 2003), petrochemicals (Williamson and Palframan, 1989), geological (Toebot et al., 1999) and foods (Hood and Zottola, 1997). Image analysis has also been used to study bacterial motility (Gualtieri et al., 1988), physiological processes in bacteria (Goldstein et al., 1988), colony counting (Corkidi et al., 1998), adhesion (Sjollema et al., 1990; Reinhard et al., 2000; Mahony et al., 2005) and bacterial rock weathering (Puente et al., 2006). Renata et al. (1999) have reported spore crops of Bacillus subtilis and Bacillus cereus with the help of electron microscopy and image analysis.

Image analysis can be, therefore, used to quantify cell size distribution (Elfwing et al., 2004). The size distribution has been shown to be changing during the growth of microorganisms. Erlebach et al. (2000) have demonstrated that the cell distribution shifts towards lower cell diameter during the course of fermentation. Although reports for cell size distribution exist, further analysis to quantify the measured cell size distribution is scarce.

Therefore, the main objective of the current work was to quantify cell size distribution during the growth of a given culture. We report the cell size distribution of Corynebacterium glutamicum (CCFT 79), a Gram-positive organism. We have studied the growth dynamics and size distribution for two conditions, (i) growth obtained from normal seed culture and (ii) growth obtained through serial dilution of seed culture. We develop a method to quantify cell size distribution using a cumulative distribution function. Further, the distribution function is used to predict an average growth property of the cell culture, which was verified experimentally.

Materials and methods

Microorganism and chemicals

The organism, C. glutamicum (CCFT 79), obtained from Coleccion Espanola De Cultivos Tipo (CECT, Valencia, Spain), was used for all the experimental work. The working culture of this organism was maintained on slant (LBG5 medium containing 5 g l⁻¹ glucose, 5 g l⁻¹ yeast extract, 10 g l⁻¹ tryptone, 5 g l⁻¹ NaCl with 2% agar) at 4 °C. The culture was revived once every month. Yeast extract and tryptone, biotin, streptomycin, amino acids were obtained from Hi-media, Mumbai, India.

Bacterial growth conditions

Seed culture

The culture was grown firstly on LBG5 media composition described by Vallino and Stephanopoulos (1993). One loop full of culture was inoculated from the slant, into the 50 ml LBG5 seed medium in a 250 ml Erlenmeyer flask. The seed culture was grown at 30 °C at 230 rpm for 10 h.

Growth from normal seed culture

A total of 10% (v/v) of the seed was transferred to the 100 ml preculturing medium (PMB) as media composition described by Vallino and Stephanopoulos (1993), in a 500 ml Erlenmeyer flask and the growth conditions were maintained as described by seed culture. OD was measured at different time and samples were taken for image analysis at various time points.

Growth from serial dilution of seed culture

In this case, the growth experiments were performed with a fewer numbers of cells obtained through serial dilution of the seed culture as described by Pelczar et al. (1993). C. glutamicum was inoculated into the seed media. At early log phase (6 h), after serial dilution (of about 10¹⁰ fold) 50 μl of the sample was transferred into the next seed media. Exponentially growing cells (at a cell OD of about 1.5–2) were diluted and further, transferred into fresh media. The same was repeated thrice and finally, 10 ml of the seed culture of OD 1.5 was transferred to the PMB. OD was measured and samples were taken for image analysis at various time points.

Cell separation protocol

Cells of C. glutamicum generally exist as a colony of two or more cells sticking together. Therefore, cell separation was essential before the measurement of size distribution. The method used for cell separation was a modified procedure developed by Noda and Kanemasa (1986). After growing the organism on the PMB, 10 ml of the sample (fermentation broth) was separated from the preculturing broth by centrifuging at 5000 rpm for 5 min. The
sample was later washed once with physiological saline solution by vortexing and centrifuging at 5000 rpm for 5 min. The cells were resuspended in 10 ml of 0.02 M phosphate buffer (pH 7.8). From this sample, 5 ml was transferred into a test tube and sonicated in a bath type sonicator (32 MHz) for 5 min. Then, 2 ml of streptomycin (0.01 g ml\(^{-1}\)) was added to the sample to arrest the cell size. After 10 minutes, 5 ml of 1000 ppm nonionic surfactant (Tween 20) was added and vortexed thoroughly and kept for 10 min. Finally, the cells were vortexed and further sonicated for 8 min. The whole procedure was performed in a 4 °C environment. Microscopic examination of the cells indicated that cells were not lysed. This protocol ensured complete separation of cells.

**Image analysis**

Image analysis was performed for cell size distribution of *C. glutamicum* after performing the above protocol to separate the cells. The cell size area was measured using an Olympus compound microscope (BX51), which was attached with Evaluation MP Cybernetics camera. Samples were taken at different fermentation time points, which were followed by the cell separation protocol. During slide preparation, special care was taken to ensure that only a monolayer of the organisms was present between slide and the cover slip by appropriate dilution of the sample (about OD of 3–3.5). Snaps of images were taken through the differential interference contrast (DIC) mode. At each time point, snaps 15 images were taken so that at least thousand cells were countable. The area in terms of \(\mu m^2\) of the individual cell was measured with the help of image analysis software (Image Pro Plus version 4.5). Image Pro Plus was able to automatically recognize clusters of brighter pixels as cells and to calculate the area of every cell and designate every cell with \(x\) and \(y\) coordinates. The data was later documented as normalized cumulative cell distribution.

**Results and discussion**

Two different seed culturing protocols were used to grow *C. glutamicum* on a growth media as described in the methods. Figure 1 shows the biomass concentration as OD measurement obtained at various time points for these two protocols. It can be noted that the maximum OD of 15.5 was observed in both the states of culture. However, growth through serial dilution achieved maximum OD at 13 h, while the normal growth obtained the same OD at about 17 h. The growth through serial dilution demonstrated a steeper rise in the exponential growth phase as compared to the normal growth. This corresponds to a higher maximum specific growth rate for the growth through serial dilution (0.67 h\(^{-1}\)) as compared to the growth rate for the normal growth (0.53 h\(^{-1}\)). It was interesting to note that the growth rates observed for both the growth conditions were different although the medium was the same. It is pertinent to raise the question regarding the influence of the cell size distribution on the growth rate for the two different states of the culture.

To address this issue, we analyzed two states of the culture by image analysis and obtained the cumulative cell size distribution with respect to the two-dimensional incident surface areas of the cells. The frame of the image of cells captures various orientations of the cells giving an average cell distribution with respect to surface area. To eliminate a bias obtained with respect to surface area, 15 such images were considered for each time point and an average cell distribution was reported. It can be noted that about 90% of the cells in the image shown in Fig. 2a are oriented in the axial direction and this minimizes the associated error (optimal orientation of the cell is indicated by ‘arrow’, while ‘cross’ indicates an orientation which contributes to higher error). The distribution was measured at various time points and was...
normalized by the total cells in the frame of image. Such a cumulative normalized cell data for the growth of cells obtained through serial dilution at 12 h is shown in Fig. 2b. The cell distribution profile demonstrates a classic S-type curve. Therefore, the normalized cumulative cell distribution (N) was represented by the following equation:

$$N = \frac{x^a}{k^2 + x^2},$$  \hspace{1cm} (1)

where ‘x’ is the incident surface area (in $\mu m^2$) of the cell, ‘k’ the half saturation constant which indicates the area attained by half of the population and ‘a’ an exponent parameter indicating the spread of the distribution. The experimental cell distribution obtained from image analysis was fitted with Eq. (1) to estimate the value of ‘a’ and ‘k’ using $f_{minsearch}$ function in MATLAB (The Math works Inc., USA). The fit for the growth through serial dilution at 12 h resulted in $a = 7.6$ and $k = 1.12 \mu m^2$. This indicates that 50% of the cells were with an incident surface area of less than $1.12 \mu m^2$ at $t = 12$ h.

Figure 3a shows the normalized cumulative cell distribution at various time points during normal growth of cells. Figure 3b shows the resulting ‘a’ and ‘k’ values obtained by fitting the data to Eq. (1). It is clear from the figure that the value of ‘a’, the exponent of Eq. (1), increases in time from 4.3 to 7, indicating that the distribution becomes steeper in time, where as the value of ‘k’, the half saturation constant decreases in time from 1.3 to 0.95 $\mu m^2$. This implies that distribution profile shifts to the left and also the cell size of the population becomes more uniform during the course of fermentation. Therefore, the percentage of cells having lower incident surface area increases along the fermentation time.

Additionally, the equation obtained to represent the cumulative distribution of cells based on their surface area (N) can be used to evaluate the normalized cell distribution (n) by differentiating Eq. (1). Such a normalized cell distribution yields the frequency for a particular cell size as a continuous function. Thus, ‘n’, the normalized cell distribution can be represented as

$$n = \frac{dN}{dt}. \hspace{1cm} (2)$$

The estimated normalized cell distribution ‘n’ at various time points are shown in Fig. 3c. It can be observed that the distribution was slightly skewed around the value of ‘k’, the half saturation constant, towards the higher surface area. The area under the curve equals unity since the cumulative distribution was normalized. Also, the number of cells with a cell size equal to the value of ‘k’ was maximum. The value of maximum frequency thus obtained at ‘k’ increases with increase in time. This indicates that the distribution was steeper at higher time points (reflected by higher value of ‘a’).

Image analysis was also performed for the growth obtained through serial dilution, which was grown on the same media as used for the normal growth, but precultured in a very different manner (see methods). The normalized cumulative cell distribution based on incident surface area is shown in Fig. 4a for growth obtained through serial dilution. In this case, the value of ‘a’ was almost constant in
the range of 7–7.6, while the value of ‘k’ lowered in time as in the case of normal growth. This indicates that the cell distribution was steeper at all times but shifts to the left during the course of fermentation (see Fig. 4b). This is consistent with the fact that the culture had the cells, which were in a similar state of division. Such a behavior is also termed as self-similar growth, wherein the cumulative distribution of cells only shifts in time but has the same steepness (indicated by the constant ‘a’ value) (Ramkrishna and Schell, 1999).

The growth of the cells obtained through serial dilution was found to be different from normal growth (Fig. 1). This difference can be attributed to the variation in cell distribution as the growth medium used had the same composition. One can predict the growth rate based on the cell division to verify this hypothesis. It was assumed that cells

![Diagram](image)

**Figure 3.** (a) The cumulative cell size distribution for normal growth as measured by incident surface area (µm²) at different time points (h) of fermentation. The distribution is shown for time points \( t = 6, 9, 12, 15, 18 \). It can be noted that the profile shifts to the left in time.

(b) Value of ‘a’ and ‘k’ obtained from fitting the distribution profile shown in Fig. 3a at various time points. Symbols indicate: ‘a’, ■; ‘k’, △. (c) The normalized cell distribution (as area in µm²) as estimated from Eq. (2) for the data shown in Fig. 3a. The estimated profiles have a peak value at incident area equal to the value of ‘k’.

![Diagram](image)

**Figure 4.** (a) The cumulative cell size distribution for cells obtained through serial dilution as measured by incident surface area (µm²) at different time points (h) of fermentation. The distribution is shown for time points \( t = 6, 7.5, 9, 10.5 \) and 12 h. It can be noted that the profile shifts to the left in time. (b) The normalized cell distribution (as area in µm²) as estimated from Eq. (2) for the data is shown in Fig. 4a. The estimated profiles have a peak value at incident area equal to the value of ‘k’.
having surface area greater than $1.6 \mu m^2$ grow at a maximum rate of $0.67 h^{-1}$, which was estimated from the maximum specific growth rate of cells growth obtained through serial dilution. Firstly, the normalized cell distribution was used to obtain the fraction of cells having surface area greater than $1.6 \mu m^2$ for culture in both the states. Figure 5a shows the histogram of the cells having greater than $1.6 \mu m^2$ for the normal growth at various time points during fermentation. At $t = 6 h$, 36% of the cells have a surface area greater than $1.6 \mu m^2$ and are assumed to be dividing maximally. And 64% of the cells, having surface area less than $1.6 \mu m^2$, are all assumed to be nondividing. It is clear from the Fig. 5a, that the fraction of cells, which are greater than $1.6 \mu m^2$, goes on decreasing in time. The fraction of such cells decreases from 36% at $t = 6 h$ to 1% at $t = 18 h$.

Based on the above fraction, the average growth rate was estimated on the assumption that the cells with surface area greater than $1.6 \mu m^2$ will have a specific rate of division equal to $0.67 h^{-1}$ (i.e. the maximum specific growth rate for the cells growth obtained through serial dilution). For example, at $t = 6 h$ since 36% of cells were dividing at the maximum rate (see Fig. 5a), the growth rate of the overall population can be estimated to be $0.24 h^{-1}$ (i.e. $0.36 \times 0.67 h^{-1}$, also see Fig. 5b). The average specific growth rate can also be obtained from the slope of the growth curve for the normal growth (see in Fig. 1) at $t = 6 h$. Figure 5b shows a comparison between estimated specific growth rates obtained from the image analysis to that obtained from growth experiments. The average specific growth rate of the culture estimated from the image analysis matched the values obtained from the growth curve. The error between the average growth rate, estimated from image analysis and from the growth experiment was in the range of 1–15%.

A similar analysis for the cells growth obtained through serial dilution (see Fig. 6a) indicate that 82% of the cells have surface area greater than $1.6 \mu m^2$ at $t = 6 h$. This fraction decreases to 7% at $t = 12 h$. There are two points to be noted: (i) cells with surface area higher than $1.6 \mu m^2$ at $t = 6 h$ obtained through growth from serial dilution of seed culture was higher (by about 55%) as compared to that observed for the normal growth; (ii) the shift from dividing to nondividing occurred during a shorter duration of time (approximately $6 h$) as compared to that of the normal growth ($12 h$). Fig. 6b shows a comparison between estimated growth rates obtained from image analysis to that obtained from growth experiment for cells growth obtained through serial dilution. In this case, image analysis had a closer match to the rate obtained from OD measurement. This was indicated by only a maximum error of 12%. The average growth rate was not only higher, but also steeper for the culture growth obtained through serial dilution indicating that the cells underwent the shift to the nondividing in a very short period.

The above assumption of dividing cells having incident surface area greater than $1.6 \mu m^2$ was verified by obtaining histogram for other incident surface area. Therefore, the above analysis was repeated for the incident surface area equal to 1.5 and $1.7 \mu m^2$. The estimated specific growth rates from such an analysis yielded higher errors implying that the $1.6 \mu m^2$ was an optimal value of incident surface area above which cell division take place (results not shown). Thus, the analysis also estimated the average cell size necessary for cell division to occur for C. glutamicum.
Cell size distribution is known to play an important role in the physiological state of cells. Past studies have shown that cell division is initiated at a critical size (Grewal and Edgar, 2003). Although coulter counter, flow cytometry and image analysis have been used to measure the cell size distribution based on size and surface area, quantification of such data is scarce. We present a quantification methodology of cell distribution based on incident surface area by using image analysis. We, further, use a distribution function to predict the possible variation in growth rate due to different seed-culturing states of the culture.

We have used a cumulative distribution function to quantify the growth of *C. glutamicum*. Image analysis yielded the cumulative distribution of cells with different incident surface area for cells obtained through normal growth and through serial dilution of seed culture. The orientation of the cells is an important issue and to counter the same several images at a given time has been used to yield an average normalized cumulative distribution. As expected, the growth obtained through serial dilution yielded a steeper cell distribution indicating that most of the cells were in a similar state of division. Whereas, in the normal growth, cells had more variation represented by a lower value of the exponent ‘α’. More cells were in the nondividing state during the course of the fermentation, which implied that the cell distribution was becoming more uniform. This was represented with an increase in the value of exponent ‘α’. It was also noted that the average cell size as measured by incident surface area was larger in exponential growth phase than in the stationary phase. The shift between these stages was shorter for the cells growth obtained through serial dilution as compared to normal growth. The cell division can also be used to yield average cell property in time. We show that the normalized cell distribution can be used to evaluate the percentage of cells that are dividing in a population.

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### References


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