Analysis of Distributed Growth of *Saccharomyces cerevisiae* Cells Immobilized in Polyacrylamide Gel

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A technique is described for the quantitative determination of the distributed growth of *Saccharomyces cerevisiae* immobilized in polyacrylamide gel. Gel specimens were embedded in paraffin or gelatin and paraffin before sectioning and staining. Photomicrographs of specimen sections were enlarged, and cell microcolony volumes were determined as a function of position in the gel by grid transparency analysis. Overall cell densities within the gel were calculated for a quantitative comparison with values measured by a second spectrophotometric method. The results show good agreement and demonstrate the sigmoidal growth of the immobilized cells, reaching a maximum steady-state value. The technique shows promise as a general method for following the transient growth of organisms immobilized within gel particles.

The immobilization of whole living cells has been shown to improve significantly ethanol productivities by yeast and bacterial fermentations (1, 5). Cell immobilization, particularly by entrapment in polyacrylamide, agar, alginate, or carrageenan gels, allows very high cell densities to be achieved, even in continuous flow systems operating above the critical dilution rate.

Recent studies of yeasts immobilized in carrageenan (11, 12), agar (J. A. Polack, W. Y. Kun, Y. K. Cho, and D. F. Day, Abstr. 74th Annu. Am. Inst. Chem. Eng. Mtg., 1981), and polyacrylamide (9) gels, and of bacteria immobilized in carrageenan gels (8), have shown that the high cell densities are a result of cellular growth within these gels and that the growth is not uniform throughout the gel particles. The growth of the immobilized cells is determined by the diffusion and concentration gradients of nutrients in and metabolic products out. The latter aspect is of particular importance when the end product of metabolism is an inhibitor of cellular growth, as in ethanol fermentation.

Electron microscopy, both scanning and transmission, has been extensively used to study the behavior and qualitative distribution of growth within the gel matrices (2, 3, 10, 12). Photomicrography of gel bead sections was used to show that the growth of *Bacillus amyloliquefaciens* was limited to the outer 50 μm of the gel beads (8). In this case, the cellular density was so low that stabilization of the specimens before sectioning was needed.

The commercial application of immobilized cell systems generally requires high cell densities. Very little quantitative information is available on the viability, growth, and product formation characteristics of cells under such conditions. In studies to date, the gel matrix-cell system has been treated in a lumped fashion, i.e., the nonuniform distribution of cell density and activity, although acknowledged qualitatively, is ignored in any quantitative modeling of such systems.

To answer questions concerning optimal conditions for cell growth and productivity requires an understanding of the distributed system. In this paper, a method is presented by which gel specimens can be stabilized under heavy cell loadings with few artifacts being introduced. In this way, a quantitative distributional analysis of growth within the gel matrix can be obtained. The system studied was *Saccharomyces cerevisiae* immobilized in polyacrylamide.

**MATERIALS AND METHODS**

**Yeast strain and growth media.** The yeast strain *S. cerevisiae* UQM 70Y was used in this work. The strain was obtained from the Microbiology Department Culture Collection, University of Queensland.

The composition of the growth medium was 100 g of glucose, 7 g of yeast extract (GIBCO Laboratories), 5 g of peptone no. 70 (GIBCO Laboratories), 5 g of (NH₄)₂SO₄, 0.3 g of KH₂PO₄, and 0.2 g of MgSO₄·7 H₂O in 1 liter of tap water. The pH was adjusted to 4.8 before sterilization by autoclaving at 105°C for 1.5 h.

**Immobilization of yeast cells.** A 50-ml yeast culture grown at 30°C to the stationary phase was spun down gently on a bench centrifuge and suspended in physiological saline (0.85% NaCl). The four polyacrylamide reagents were also prepared in physiological saline as...
follows: the major monomer, 47.5% acrylamide (BDH); the cross-linking monomer, 2.5% N,N-methylene bisacrylamide (BDH); the catalyst, 2.5% N,N,N',N''-tetramethylethylenediamine (Sigma Chemical Co.); and the initiator, 2.5% potassium persulfate.

To make a 10% polyacrylamide gel with immobilized cells, 5 ml of each of the two monomer reagents, 1 ml of the catalyst, 2 ml of the initiator, and 5 ml of the yeast suspension were added to 7 ml of saline, the mixture being sparged with nitrogen gas to remove oxygen, which interferes with the free radical reaction. The mixture was immediately poured into stainless steel trays to give a depth of 1 mm and then was left to set into a gel for 10 to 15 min. The trays were kept in an enclosed water bath (25°C) which was flooded with nitrogen gas. The resulting gels were cut into slabs (10 by 10 by 1 mm) and washed several times in physiological saline to remove unreacted toxic monomers and any free yeast cells.

Growth studies were performed by placing the gel slabs in a temperature-controlled (30°C), well-mixed, continuous culture system which contained the growth medium and which was operated at a dilution rate well above the critical dilution rate for yeast. The high dilution rates (ca. 0.8 h⁻¹) ensured high and relatively constant substrate concentrations, minimized the number of free yeast cells arising from gel leakage, and (together with the low pH of the growth medium) minimized the chance of contamination of the fermentor culture.

**Average cell density determination.** Gel slabs were removed from the fermentor at regular intervals. The two long dimensions of the gel slab were measured with a ruler (±0.1 mm); the thickness of the slab was measured by taking a cross-sectional cut and measuring it microscopically with the aid of a calibrated ocular grating (±0.03 mm).

All the pieces of the gel slab were then ground to a paste with a porcelain mortar and pestle, and the paste was diluted to a known volume for turbidity measurements with a spectrophotometer set at 540 mm. Absorbance readings were corrected for the polycrylamide gel, and cell densities for the gel volume were calculated. These were obtained by converting absorbance units to grams (dry weight) of cells per liter of gel via a standard curve, i.e., grams (dry weight) of cells per liter = (A₅₄₀ - A₅₄₀gel) × 0.18, where A is absorbance.

**Gel fixation and embedding.** At the same time when average cell density determinations were made, a second gel slab was removed for photomicrographic analysis. These slabs were fixed in a 4% Formalin-2.5% glutaraldehyde saline solution for more than 12 h after which the fixative was removed by several washings in 0.67 M cacodylate buffer.

If gelatin embedment was used, the lab specimens were immersed in 10% gelatin overnight at 37°C and then were cooled and hardened in 10% Formalin overnight. Additional growth did not occur since the fixative is toxic to cell growth. The gelatin specimen blocks were trimmed and then treated in the same manner as the non-gelatin-embedded specimens as described below.

The specimens were dehydrated in a rapid ethanol-distilled water series of 70, 95, and 3 × 100% ethanol solutions for 30 min each. The ethanol was replaced with xylene by moving the specimens through two changes of 100% xylene for 30 min each. The xylene was then replaced by paraffin wax by three changes in 100% paraffin for 30 min each in a vacuum oven (70 kPa, 58°C). The paraffin-infiltrated specimens were then embedded in a paraffin block, which was cooled for 30 min at 10 to 15°C and then placed in a refrigerator (4°C) overnight before sectioning.

**Sectioning.** The rotary microtome was used to face off the paraffin specimen blocks, and then 5 to 7 μm sections were cut and floated onto a 48°C water bath. The specimen sections were floated onto glass slides which had been lightly smeared with egg albumin and dried overnight at 56°C at an incline to remove excess paraffin.

**Staining.** Before staining, the wax was removed completely, and the section was brought down to water. The method employed was two transfers in 100% xylene for 15 min each, absolute ethanol (two changes, 2 min each), 90% ethanol (2 min), 70% ethanol (2 min), 50% ethanol (2 min), water.

The hydrated specimens were stained with 1% toluidine blue in 1% aqueous borax for approximately 30 s, washed with distilled water, and then dehydrated through a series of ascending concentrations of alcohol, cleared in xylene, and mounted under a cover slip with Depex, which was allowed to harden for several hours.

**Photomicrographic Analysis.** Photomicrographs were taken of the specimen sections, and then the negatives were enlarged to produce prints of 17 by 25 cm. The relative volume of cells was determined as a function of distance from the gel surface by superimposing a fine-mesh grid transparency onto the enlargement prints. The grid area analyzed varied from 40 by 80 to 40 by 130 grid units, depending on the specimen sizes, and the darkly stained cell colony volumes were calculated by counting grid areas to the closest one-half grid area.

From these data, a histogram was plotted as relative cell volume versus relative distance from one gel surface. The data were smoothed by using a least-squares polynomial regression of the form

\[ y = \sum_{i=0}^{n} a_i x^i \]

where y is the relative cell volume and x is the relative distance from one gel surface. By using the thickness measurements made in the average cell density determinations, the relative distance and volume measurements are converted to absolute values.

The average cell densities were then calculated by integrating the above relation from one surface to the other for each gel section and by assuming that hydrated cells contain approximately 80% water with a specific gravity ratio of 1.13 with respect to water. This calculation of average cell density over the whole gel slab neglects any end effects on the cell density distribution around the sides of the slab.

**RESULTS AND DISCUSSION**

**Specimen preparation and photomicrography.** The photomicrographs in Fig. 1 show the progression of yeast growth within the gel slabs with increasing incubation time. The preparation of the specimens in Fig. 1B and D included the
gelatin embedding steps, whereas preparation of the remaining specimens shown in Fig. 1 did not. As indicated by these photomicrographs, embedding in gelatin commonly resulted in greater stain retention by the specimen sections, thus reducing the contrast between the cell microcolonies and gel matrix.

Gelatin is frequently used in histopathological preparations to stabilize friable or fragmented tissues but is known to stain quite strongly with basic anilin dyes (6). The advantage to be gained by gelatin embedding was that the specimens were not subject to as much compression upon sectioning as were those specimens not enclosed in gelatin. In those specimens from the latter part of the experiment, in which the gel samples were densely packed with cells, the gelatin also ensured that the loosely bound layer of cells of the gel surfaces remained intact.

To prevent the gelatin from peeling away from

FIG. 1. Stained sections showing the progression of immobilized cell growth with increasing incubation time. (A) 0 h; (B) 21 h, specimen gelatin embedded; (C) 25 h; (D) 30 h; (E) 46.5 h, specimen gelatin embedded; (F) 69 h. Bars, 350 μm.
the section, the gelatin must be allowed to penetrate the specimen. For this reason, the fixative reagents must be thoroughly washed from the specimens; otherwise, they will harden the gelatin before it is able to penetrate completely.

A combination of the two reagents, formaldehyde and glutaraldehyde, was used for specimen fixation. Formaldehyde penetrates the specimens quickly for faster fixation but is rather soft fixative, whereas glutaraldehyde gives greater hardening but does not have the same penetration as formaldehyde. The fixatives effectively bind the gel-cell specimens with only an occasional loss of cells owing to sectioning (Fig. 1C and D). In the quantitative analyses of the photomicrographs, any hollow microcolonies, which were clearly caused by cell loss on sectioning, were counted as being full of cells.

The need for an infiltrating and embedding matrix such as paraffin for successful sectioning becomes obvious when the extensive channeling in Fig. 1F is observed. This channeling was a result of gel and colony disruption caused by cellular growth and forced CO$_2$ evolution. The vacuum infiltration used here helps to remove the gases from the cavities within the gels and permits their filling with paraffin. After sectioning, the paraffin is subsequently removed. If it is felt that continuous section support is necessary, embedding in gelatin is advisable. Quite a degree of section folding was encountered with many of the paraffin-infiltrated specimens as they were floated onto slides after sectioning. Gelatin embedding before paraffin infiltration largely eliminated the problem of section folding. Folding was not a significant problem in the quantitative analyses since several sections were taken from each specimen, and only a portion of each specimen section was required for analysis.

The rapid ethanol-water dehydration series used here was chosen after it was shown that the extended dehydration series, or long storage in ethanol, caused the specimens to become hard and brittle. Similarly, storage in xylene should be minimized as this also resulted in specimens too brittle for clean sectioning.

![Graph](image-url)

**FIG. 2.** Histogram and polynomial smoothing of grid data ($t = 30$ h). The relative number of grid units refers to the number of lines on the ocular grating. The innermost position of the gel occurs at a value of approximately 45 grid units.

<table>
<thead>
<tr>
<th>Sampling time (h)</th>
<th>Polynomial relation*</th>
<th>Vol % cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$Y = 19.9 - 1.30X + 0.29 \times 10^{-1}X^2 - 1.78 \times 10^{-4}X^3$</td>
<td>11.0</td>
</tr>
<tr>
<td>21</td>
<td>$Y = 26.0 - 0.70X + 1.81 \times 10^{-6}X^4$</td>
<td>14.2</td>
</tr>
<tr>
<td>25</td>
<td>$Y = 27.1 - 1.95 \times 10^{-2}X^2 + 4.56 \times 10^{-9}X^4 - 4.22 \times 10^{-14}X^6$</td>
<td>23.3</td>
</tr>
<tr>
<td>30</td>
<td>$Y = 50.7 - 1.12X + 1.02 \times 10^{-7}X^3 - 9.76 \times 10^{-10}X^6$</td>
<td>35.9</td>
</tr>
<tr>
<td>46.5</td>
<td>$Y = 19.4 + 0.57X - 1.18 \times 10^{-2}X^2 + 3.17 \times 10^{-12}X^7 - 2.44 \times 10^{-14}X^9$</td>
<td>59.0</td>
</tr>
<tr>
<td>69</td>
<td>$Y = 19.4 + 0.57X - 1.18 \times 10^{-2}X^2 + 3.17 \times 10^{-12}X^7 - 2.44 \times 10^{-14}X^9$</td>
<td>56.3</td>
</tr>
</tbody>
</table>

* $Y$, Relative cell volume from 0 to maximum of 40; $X$, relative distance across gel cross sections; $X^9$, $X$ values representing the gel surface.
Quantitative analysis. Enlargements of photomicrographs similar to those in Fig. 1 were used to create the cell population histograms for the sampling times of 21, 25, 30, 46.5, and 69 h. The histogram for 30 h (Fig. 2) is shown as an example to illustrate the relationship between the histograms and polynomial smoothing of the grid analysis data. The equations, describing the polynomial approximations of the cell distributions in each of the above sampling times, are presented in Table 1.

Integration of these polynomial equations between the limits describing the gel surfaces leads to a value for the volume percentage of cells in each specimen (Table 1). To assess the quantitative accuracy of this analytical method, the cell volume percentages were converted to biomass density values, as described above. These values are then compared to those calculated by the average cell density determination method (Fig. 3).

One of the assumptions in the calculation of the overall biomass density by the photomicroscopic technique is that the microcolonies have a constant cell density determined by their maximum packing configuration. In high-magnification observations of the specimen sections, it was shown that this was not always true in specimens from the latter part of the experiment (Fig. 4). The principal reason for this is that after prolonged incubation, the gels lose much of their mechanical rigidity owing to gel rupture, particularly near the gel surfaces, where cellular growth and CO₂ evolution is maximal. These differences in microcolony density are probably the cause of overestimates in the biomass densities at 46.5 and 69 h because the photomicrographic determinations were based on microcolony volume and not on cell concentration, as in the average cell density technique.

The results from both methods generate the sigmoidal growth curve for the immobilized cells (Fig. 3), as has been reported by others (4, 12). The biomass reached a maximum value, by which time the gels had increased in volume by a factor of over threefold. This maximum cell density represents a steady state reached between cellular growth within the gel matrix and cell leakage from the, by now, disrupted gel. Cell leakage was evident from the contamination of the surrounding media.

In summary, this photomicrographic method has provided a means by which the dynamics of the distributed growth of immobilized cells can be quantified further than the general description that growth predominates at the surfaces of the gels. By utilizing the smoothed equations given in Table 1, the cell density can be determined as a function of location. Alternatively, the relative volume occupied by cells in different regions of the gel can be calculated by integrating between the appropriate limits.

With the aid of such techniques as viability stains (7), it is hoped that it will be possible to superimpose viability profiles onto the distributed growth profiles described here. This will lead to an effective method of modeling immobilized cell growth, which will thus allow prediction of optimal gel sizes and shapes for production of compounds such as ethanol.

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LITERATURE CITED