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Modeling the Interactions of *Lactobacillus curvatus* Colonies in Solid Medium: Consequences for Food Quality and Safety

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The growth process of *Lactobacillus curvatus* colonies was quantified by a coupled growth and diffusion equation incorporating a volumetric rate of lactic acid production. Analytical solutions were compared to numerical ones, and both were able to predict the onset of interaction well. The derived analytical solution modeled the lactic acid concentration profile as a function of the diffusion coefficient, colony radius, and volumetric production rate. Interaction was assumed to occur when the volume-averaged specific growth rate of the cells in a colony was 90% of the initial maximum rate. Growth of *L. curvatus* in solid medium is dependent on the number of cells in a colony. In colonies with populations of fewer than 10^5 cells, mass transfer limitation is not significant for the growth process. When the initial inoculation density is relatively high, colonies are not able to grow to these sizes and growth approaches that of broth cultures (negligible mass transfer limitation). In foods, which resemble the model solid system and in which the initial inoculation density is high, it will be appropriate to use predictive models of broth cultures to estimate growth. For a very low initial inoculation density, large colonies can develop that will start to deviate from growth in broth cultures, but only after large outgrowth.

The mathematical description of growth responses of microorganisms has a long history. In the recent 2 decades, however, a new family of these models has been developed for prediction of food quality and safety. These are usually multidimensional activator and inhibitor empirical models for predicting growth of microorganisms. The independent variables are the environmental conditions of the food in which these microorganisms grow. As the demand for minimally processed foods increases, these models will play an increasingly important role in ensuring the quality and safety of these foods.

Almost all of these models are derived from experiments with liquid cultures, where the environmental conditions are homogeneous. However, there are many types of foods where this is not the case. One example is growth of microorganisms in sausages (4). Microorganisms grow as colonies or nests in these foods, and microgradients can occur in and around these colonies. The microgradients are caused by diffusive transport of substrate into the colonies and metabolic products out of the colonies. Microgradients of pH were detected in colonies of *Salmonella enterica* serovar Typhimurium with microelectrodes (14) and in colonies of *Lactobacillus curvatus* by the fluorescence ratio imaging technique (7). In time, these microgradients should produce a set of varying specific growth rates in a colony.

These mass transfer effects can be important for predicting spoilage and safety. The strategy of controlling the growth of this pathogen in this heterogeneous system might be different from that used for the free-living species. The aim of this paper is to quantify these mass transfer effects in a model system. This model system consists of a homolactic bacterium, *L. curvatus*, growing in a nutrient medium that has been solidified with agar. *L. curvatus* is a typical meat spoilage microorganism.

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THEORY

In our model system, the solid matrix restricts the movement of individual cells or groups of cells of *L. curvatus* (nonmotile) and forces these cells to grow as individual colonies. The colony grows by addition of cells, and we assume a maximum closed packing density of $\sim 10^{17}$ cell/m^3 (3, 10). This is a reasonable assumption, as these nonmotile cells will use all of the available space to grow in this matrix. Furthermore, there will be diffusion of the substrate and metabolic end products in and out of the colony. The substrate used is glucose (S, in moles per cubic meter), and the main metabolic end product is lactic acid (P, in moles per cubic meter). The lactic acid produced lowers the pH.

Growth kinetics. The population growth of an isolated spherical colony after a lag period of λ can be represented by the differential equation

$$\frac{d(nV)}{dt} = \mu_m \cdot (nV)\ (t > \lambda)\ (\text{cells/s}) \quad (1)$$

where

$$\mu_m = f(P, S, \text{pH})_{r,t}\ (1/\text{s}) \quad (2)$$

TABLE 1. Parameter values for equations 1, 3, 5, 6, and 7 for *L. curvatus*

Equation(s)	Parameter	Reference	Value
1	n	3	10^{17} cells/m^3 or 10^{11} cells/ml[a]
3	μ_{opt}	15	$2.94 \cdot 10^{-4}$/s or 1.06/h
3	K_s	13	$4 \cdot 10^{-4}$ mol/m^3
3	b_2	8	$3.89 \cdot 10^{-7}$ m^3/mol/s or $1.40 \cdot 10^{-3}$ m^3/mol/h
3	pH_{min}	15	4.26
3	pH_{max}	15	9.77
6	Y_{XP}	8	$3.3 \cdot 10^{13}$ cells/mol
6	m_P	11	$1.3 \cdot 10^{-19}$ mol/cell/s
5, 7	D_P	—[b]	$2.81 \cdot 10^{-10}$ m^2/s

[a] We assume that one cell equals 1 CFU.
[b] —, P. K. Malakar, M. H. Zwietering, R. M. Boom, T. F. Brocklehurst, P. D. G. Wilson, A. R. Mackie, and K. van't Riet, submitted for publication.

and n (cells per cubic meter) is the cell packing density, V (cubic meters) is the volume of the colony, and μ_m (1/s) is the local specific growth rate. The subscripts r and t refer to the dependence of μ_m on P, S, and pH at a certain radial distance and time period.

The specific growth rate of *L. curvatus* in broth culture, μ_m (8), a function of S, pH, and P, can be described by

$$\mu_m = \mu_{opt}\left(\frac{S}{K_s + S}\right) \left[\frac{4(pH - pH_{min})(pH_{max} - pH)}{(pH_{max} - pH_{min})^2}\right] - (b_2 P)\ (1/s) \quad (3)$$

where μ_{opt} (1/s) is the growth rate when conditions are optimal. This occurs when S is much greater than K_s (moles per cubic meter), P is zero, and the pH is equal to the midpoint value between pH_{max} and pH_{min}. The parameter K_s is a Monod constant, pH_{min} is the minimum pH, and pH_{max} is the maximum pH at which no growth is observed. Finally, b_2 (cubic meters per mole per second) is a parameter describing the direct effect of P on the specific growth rate of *L. curvatus*. The fitted value of parameter b_2 was found to be independent of the initial pH of the broth culture. The numerical values of these parameters are given in Table 1.

Apart from the direct effect, P also indirectly influences the specific growth rate by decreasing the pH. The pH can be empirically related to the amount of P present by using the titration equation of Martens et al. (8), which is expressed by

$$pH = \frac{pH_0 + a_1[P]}{1 + a_2[P]} \quad (4)$$

where the parameter pH_0 is the initial pH of the culture medium and a_1 and a_2 (both in cubic meters per mole) are fitted parameters. These parameters are given in Table 2.

Equation 3 can be simplified by assuming pH and P to be the most important factors affecting growth. The low K_s value (Table 1) and abundant substrate available cause the term in equation 3 containing S to be unity. Since the pH is linked to P (equation 4), modeling of P in the colony should be sufficient to describe the growth of the colony.

Diffusion. In our model system, these growth equations (equations 1 to 3) should also be applicable. However, we will have to account for diffusive transport of P in the growth process. Using Fick's second law in spherical geometry, accumulation of lactic acid inside a colony can be represented by

$$\frac{\partial P_i}{\partial t} = D_P\left(\frac{\partial^2 P_i}{\partial r^2} + \frac{2\partial P_i}{r\ \partial r}\right) + q(r, t)\ r \le R_{col}\ (\text{mol/m}^3\text{/s}) \quad (5)$$

where the first expression on the right side is the diffusion term and the second term represents the volumetric production rate, q (moles per cubic meter per second), of the *L. curvatus* cells in the colony. The subscript i refers to location inside the colony, D_p (square meters per second) is the effective diffusion coefficient of lactic acid and R_{col} is the colony radius. The volumetric production rate can be adequately represented (5) by the expression

$$q = \left(\frac{\mu_m}{Y_{XP}} + m_p\right)n\ (\text{mol/m}^3\text{/s}) \quad (6)$$

This model consists of a growth-related and a non-growth-related term. The yield value Y_{XP} (cells per mole) is the maximum amount of biomass produced given an amount of lactic acid produced concurrently, and the non-growth-related term is represented by the maintenance coefficient m_p (moles per cell per second). Outside the colony, there is no production of lactic acid and using Fick's second law for spherical geometry again results in

$$\frac{\partial P_o}{\partial t} = D_P\left(\frac{\partial^2 P_o}{\partial r^2} + \frac{2}{r}\frac{\partial P_o}{\partial r}\right) r > R_{col}\ (\text{mol/m}^3\text{/s}) \quad (7)$$

where we assume that the effective diffusion coefficient of lactic acid (D_p) remains the same. The subscript o refers to location outside the colony.

In their paper on submerged bacterial colonies, Wimpenny et al. (16), have shown that a colony expands exponentially until a certain size is reached. Growth then proceeds linearly and comes to a stop, which they postulated might be due to by-product inhibition. The coupled growth and diffusion equations above should be able to represent this. Qualitatively, when the colony expands to a certain size, lactic acid will start to accumulate in the center, as diffusion cannot get rid of the lactic acid fast enough. Accumulation of lactic acid and the concurrent decrease in pH will result in a distribution of growth rates in the colony. Overall growth slows down and effectively stops when the pH in the whole colony and its surroundings is below the minimum pH necessary for growth.

Boundary conditions. Equations 1, 5, and 7, with the help of equations 3, 4, and 6, can be solved by using the appropriate initial and boundary conditions. We know that foods harbor more than one colony. Lactic acid diffusing away from a colony might affect a neighboring colony, so an appropriate boundary

TABLE 2. Regression parameters (6) of titration curves (in MRS broth) at initial pHs of 7 and 6

Parameter	pH_0	Value (m^3/mol)
a_1	7	0.038
a_2	7	0.015
a_1	6	0.029
a_2	6	0.011

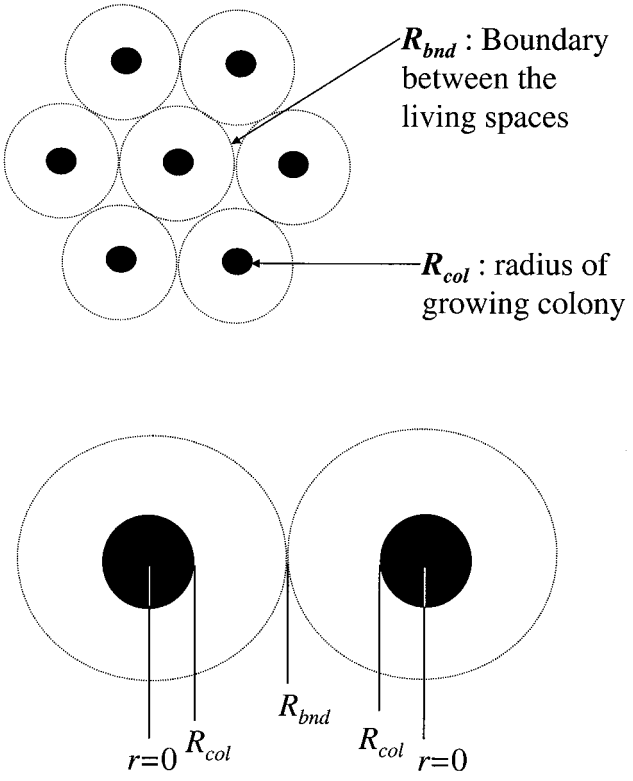

FIG. 1. Geometrical translation of the colony growth model. Each colony is sequestered in its own space, which is divided into two regions. The first is a zone of colony growth bounded by R_{col} (colony radius), which expands as new cells are added. The second is a zone of living space bounded by R_{bnd}, which is dependent on the initial inoculation density.

is at R_{bnd}, which is half of the distance between the centers of two neighboring colonies, as shown in Fig. 1 (12).

Figure 1 depicts an idealized geometry of colony distribution. Each of the colonies has a radius, R_{col}, and we assume that these colonies are homogeneously distributed in the sample. The boundaries of the living spaces are fixed at R_{bnd} by the initial inoculation density. This distance, R_{bnd}, can be calculated by assuming that the gel matrix is made up of identical cubes. Each cube contains a spherical colony in the center. A higher inoculation density means that there will be more and smaller cubes present and vice versa. The distance R_{bnd} is then one-half of the cube root of the volume of each cube,

$$R_{bnd} = \frac{1}{2}\sqrt[3]{\frac{10^{-6}}{X_0}} \text{ (meters)} \tag{8}$$

where X_0 (CFU per milliliter) is the initial inoculation density. Each colony starts growth from one cell, and in time, the radius of the colony, R_{col}, can be calculated from the number of cells present because the packing density is known. We further assume that all of the colonies behave similarly and that the total number of cells in our system is just the summation of the number of cells in every individual colony.

The boundary conditions for equations 5 and 7 at the center of the colony and at R_{bnd} are

$$\left(\frac{\partial P_i}{\partial r}\right)_{r=0} = 0 \text{ (mol/m}^3\text{/m)} \tag{9}$$

and

$$\left(\frac{\partial P_0}{\partial r}\right)_{r=R_{bnd}} = 0 \text{ (mol/m}^3\text{/m)} \tag{10}$$

because of symmetry at these points (1). Additionally the concentration and flux at the common boundary at R_{col} are

$$P_i(R_{col}) = P_o(R_{col}) \text{ (mol/m}^3\text{)} \tag{11}$$

and

$$\left(\frac{\partial P_i}{\partial r}\right)_{R_{col}} = \left(\frac{\partial P_o}{\partial r}\right)_{R_{col}} \text{ (mol/m}^3\text{/m)} \tag{12}$$

These boundary conditions and the zero initial concentration of lactic acid are sufficient to solve coupled differential equations 1, 5, and 7.

Numerical solution. These equations will be solved by using an implicit finite difference scheme with the appropriate time and distance discretization to ensure a stable solution. Note that all of the parameters in the equations are constants and not functions of either distance or time.

Analytical solution. The numerical scheme is helpful because it can simulate the total growth period of *L. curvatus*. But, on the other hand, it is not possible to get an intuitive feel for the growth of *L. curvatus* and a relatively complex calculation scheme is necessary. An analytical solution should help because the function can be evaluated easily and characteristic numbers can be derived.

A possible analytical solution to the diffusion equations follows from a pseudo-steady-state approach. Diffusion gets rid of material rapidly at small distances, and in this region, conditions change instantaneously. For a sphere of radius R, the relative amount of material that has diffused, E, is a function of the dimensionless Fourier number Fo (1) given by

$$E = 1 - \frac{6}{\pi^2}\exp(-\pi^2 Fo) \tag{13}$$

Using equation 13, the amount remaining is only 23% when $Fo = 0.1$. At a distance of 10μm and with a diffusion coefficient of 10^{-10} m^2/s, this occurs in 0.1 s. At 1 mm and with the same diffusion coefficient, it takes 17 min and at 1 cm it takes 27 h. By comparing the time constants of diffusion and growth, we can define a region where a pseudo-steady-state condition is valid. Here during the time of growth (greater than the time of diffusion), conditions remain essentially the same because most of the material has already diffused away.

Diffusion can be assumed to be much faster than growth by setting the characteristic time for diffusion (τ_D) at 1/200 of the characteristic time of growth (τ_G). By using the equation in Table 3, row 2, column 2, and filling in the values of τ_D and D_p (Table 1), the distance, R_{st}, for which this condition is valid is ~200 μm. In this region, we assume that a pseudo-steady state exists. At this range, at a certain time point, diffusion levels out

TABLE 3. Time constants of growth and diffusion[a]

Time constant(s)	Equation used	Reference
$\tau_g = 3{,}600$	$1/\mu_m$	15
$\tau_d = 18$	$D_P\tau_d/R_{st}^2 = 0.1 = Fo$	2

[a] μ_m is the specific growth rate of *L. curvatus* at 30°C. D_P is the effective diffusion coefficient of lactic acid. R_{st} is the maximum distance where the time constant of diffusion (τ_d) is 1/200 of the time of growth (τ_g).

production instantaneously. By using the pseudo-steady-state formalism, equations 5 and 7 can be written as

$$D_p\left(\frac{d^2P_i}{dr^2} + \frac{2}{r}\frac{dP_i}{dr}\right) = -q \; r \leq R_{col} \; (\text{mol/m}^3\text{/s}) \qquad (14)$$

and

$$D_p\left(\frac{d^2P_o}{dr^2} + \frac{2}{r}\frac{dP_o}{dr}\right) = 0 \; r > R_{col} \; (\text{mol/m}^3\text{/s}) \qquad (15)$$

which are second-order ordinary differential equations.

In order to solve these equations, the boundary condition

$$P_{r=\infty} = P_{t=0} = 0 \; (\text{mol/m}^3) \qquad (16)$$

can be used instead of the boundary condition described by equation 10. Initially, the concentration of P at the living space boundary R_{bnd} will not change at all (equation 10) and the analytical solution will be a good approximation. All other initial and boundary conditions remain the same. Note that the volumetric production rate, q, in equation 14 has been further simplified by assuming that it is constant. This is valid as long as the amount of lactic acid produced is too small to cause a change in pH. If there is no change in pH, the specific growth rate remains unchanged also.

The general solutions of equations 1, 14, and 15 are presented in Table 4. They contain four unknowns, *a, b, c*, and *d*, which are integration constants. Application of the boundary condition and initial conditions (Appendix A) yields

$$P_i = \frac{q}{2D_p}\left(R_{col}^2 - \frac{r^2}{3}\right) r \leq R_{col} \; (\text{mol/m}^3) \qquad (17)$$

and

$$P_o = \frac{1}{3}\frac{q}{D_p}\frac{R_{col}^3}{r} \; r > R_{col} \; (\text{mol/m}^3) \qquad (18)$$

where the concentration of lactic acid inside the colony increases with the square of the colony radius while the concentration of lactic acid outside the colony increases with the cube of the colony radius. Note that R_{col}^3 in equation 18 increases exponentially in time (Appendix C) and therefore when colonies are in close proximity, saturation of the surroundings occurs much faster.

TABLE 4. Analytical solutions of the pseudo-steady-state condition

Equation no.	Analytical solution[a]	Units
1	$N_t = \exp(\mu_m t_a)$	Cells per colony
14	$P_i = -(q/D_p)\,(r^2/6) - (a/r) + b$	Moles per cubic meter
15	$P_o = c - d/r$	Moles per cubic meter

[a] N_t is the number of cells in a colony, which develops from one cell, and t_a is time of growth. *a, b, c*, and *d* are the integration constants. μ_m is the specific growth rate of *L. curvatus* at 30°C. q is the volumetric production rate of lactic acid. D_p is the effective diffusion coefficient of lactic acid.

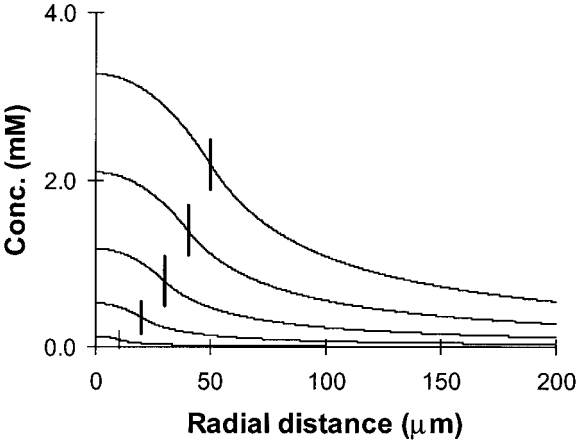

FIG. 2. Development of concentration (Conc.) profiles of lactic acid (P) in and around colonies of different sizes (radii of 10, 20, 30, 40, and 50 μm) using the pseudo-steady-state analytical solutions (equation 17 and 18). The volumetric production rate, q, and effective diffusion coefficient, D_p, were held constant at 0.78 mol/m^3/s and 10^{-10} m^2/s. The bars indicate the positions of the colony radii.

Figure 2 shows the results of using the above analytical solutions. The parameters q and D_P were kept constant, but the radius of the colony, R_{col}, was increased for each curve. The vertical bars (at 10, 20, 30, 40, and 50 μm) indicate the locations of the colony radii. Qualitatively, it shows that as the radius of the colony increases, the difference between the concentrations at the center and the radius also increases. The difference in concentration produces a set of varying local specific growth rates (μ_m) in the colony, which was postulated in the introduction. The validity of this pseudo-steady-state approach will be compared to the numerical approach.

Interactions. These analytical solutions can also give an easy and quick indication of the magnitude of the interaction time. The dimensionless quantity produced by the following equation

$$\frac{\bar{P}}{P(r = R_{col})} = 1.2 \qquad (19)$$

results from dividing the volume-averaged concentration of lactic acid in a colony, $\bar{P}$, by the concentration of lactic acid at R_{col} (Appendix B) and leads to

$$\bar{P} = 1.2\,\frac{q}{3D_p}R_{col}^2 \; (\text{mol/m}^3) \qquad (20)$$

We next define the interaction occurring when the specific growth rate is 90% of its initial maximum value (μ_m). The concentration of lactic acid when this occurs (P_{int}) can be calculated from equations 3 and 4 (Appendix B). Substituting for R_{col} (Appendix C) and P_{int} ($P_{int} \sim \bar{P}$) in equation 20 results in

$$P_{\text{int}} = 0.4\,\frac{q}{D_p}\left[\frac{0.75}{\pi}\,\frac{\exp(\mu_m t_a)}{n}\right]^{\frac{2}{3}}\ (\text{mol/m}^3) \qquad (21)$$

and

$$t_a = \frac{1}{\mu_m}\left[\frac{3}{2}\ln\left(\frac{2.5}{q}P_{\text{int}}\,D_p\right) + \ln\left(\frac{4\pi\,n}{3}\right)\right]\ (\text{seconds}) \qquad (22)$$

where t_a is the time of interaction when a lag is absent. Note that we still use μ_m in equations 21 and 22, as no analytical solution is possible if μ_m changes. This is a valid assumption since μ_m has only changed by 10%. The required approximate time of interaction, t_{int}, with a lag period is

$$t_{\text{int}} = \lambda + t_a\ (\text{seconds}) \qquad (23)$$

where λ is the lag time.

MATERIALS AND METHODS

Experimental technique. Frozen cultures of *L. curvatus* stored at −80°C were thawed and precultured at pH 5.8 (normal pH of MRS broth [Oxoid]) in MRS broth at 30°C for 22 h (start of the stationary phase). Serial dilutions were then made to obtain the desired initial inoculum density, X_0 (CFU per milliliter), for the start of the experiment. The initial inoculation densities were 1, 10, and 100 CFU/ml, and the initial pH of the growth medium was poised at 6 and 7.

A 0.3% (wt/vol) MRS agar gel was prepared by dissolving the appropriate amount of MRS powder and 3 g of bacteriological agar in 1 liter of demineralized water. A 0.3% (wt/vol) MRS agar gel was chosen because the colonies growing at this concentration were quite spherical in shape. Also, the inoculum could be introduced and mixed into the gel at around 35°C before being dispensed into petri dishes. It still remains fluid at this temperature. Normal 1% (wt/vol) agar has to be dispensed at about 50°C, and the *L. curvatus* cells might be heat stressed at that temperature.

The MRS agar was then autoclaved and kept in a water bath at 50°C. Prior to inoculation, the 0.3% (wt/vol) MRS agar was cooled to 35°C and the pH was adjusted to the initial pH of the experiment by adding 5 M NaOH or HCl. An appropriate amount of X_0 was added, and the agar solution was stirred with a magnetic stirrer to make sure that the *L. curvatus* cells were well mixed and evenly distributed. An agar dispenser was subsequently used to distribute 35 ml of the medium in 90-mm petri dishes and allowed to solidify. In an experimental batch, about 24 petri dishes were used. The petri dishes were incubated at 30°C for the duration of the experiment.

Sampling was done by stomaching. The contents of a petri dish were first weighed and increased to 100 g with a peptone solution. The resulting solution was then stomached for 3 min. An appropriate amount of this solution was then serially diluted and pour plated in standard MRS agar to enumerate the microorganisms.

Broth culture studies using the same inoculum density and temperature were performed in parallel. The experimental procedure of broth culture studies using *L. curvatus* was detailed previously (6).

Numerical solution. The numerical solutions of equations 1, 5, and 7 were accomplished in a sequential manner. We used an implicit finite difference scheme in equations 5 and 7 to calculate the concentrations of lactic acid from the center of a colony until the boundary. This scheme was continued for 0.1 h. Note that this 0.1 h is not the same as the discretized time step of the finite difference method, which is much smaller. During this period, the volumetric production rate, q, on every grid element was assumed to be constant, as was the specific growth rate, μ_m. This is reasonable since the specific growth rate, μ_m, is about 1 (1 per hour).

At the end of this 0.1-h period, the local specific growth rates in the colony were updated by using equation 3. These new local specific growth rates were then used in equation 1 to calculate the new number of cells in the colony. In this scheme, the colony expands only by a discretized amount and the packing density at the outermost layer was allowed to vary from 1 to a maximum of 10^{17} cells/m^3. The other discretized layers have a constant packing density of 10^{17} cells/m^3.

The new number of cells is then redistributed, resulting in a new packing density in the outermost layer. This information was then used to update the volumetric production rates. With the new specific growth rates, new production rates, and radius, the cycle was repeated to find the next concentration profile in the system. This was continued for a period of 60 h.

TABLE 5. Living space boundaries for various initial inoculation densities, according to equation 8

X_0 (CFU/ml)	R_{bnd}	
	Meters	Micrometers
1	0.005	5,000
10	0.0023	2,321
100	0.0011	1,077

Analytical solution. Table 5 gives the values of the boundaries of the living space (R_{bnd}) for inoculation densities of 1, 10, and 100 CFU/ml. Even though these distances are greater than 200 μm, the analytical solutions can still be used to predict the initial growth of colonies at these inoculation densities within this pseudo-steady-state distance scale.

The same sequential technique was used for the analytical pseudo-steady-state solutions since these equations are only valid for a constant q and μ_m. Every 0.1 h, the lactic acid concentration profile was calculated by using equations 17 and 18. A new volume-averaged growth rate, $\bar{\mu}_m$, had to be calculated (Appendix D) since the concentration profile was continuous. This $\bar{\mu}_m$ was then used in equations 1 and 6 to find the new number of cells and the new volume-averaged production rates. The packing density was set to 10^{17} cells/m^3 again, and from the number of cells present, the new radius was calculated. This cycle was repeated for a period of 60 h or until the colony radius reached 200 μm. The simulations were performed by using the software program Matlab from Mathworks Inc.

Lag. The lag time for all of the simulations was found by fitting the growth data of the solid medium containing an initial inoculation density of 100 CFU/ml. This was because the samples containing 1 and 10 CFU/ml did not have any data points from the start until about 10 h. We assumed the lag time to be the same for all of the samples as the initial conditions and cell history were similar except for the initial inoculation density. The modified Gompertz function (17) was used for this purpose. The lag time was found by fitting the data points of the 100-CFU/ml experiments and using a fixed μ_m. This μ_m was first calculated by using equation 3.

RESULTS AND DISCUSSION

Steady-state assumptions. Figure 3 shows a comparison of the pseudo-steady-state solution and the numerical solution for colonies with radii of 1.6 and 20 μm. In Fig. 3a, the curves of the numerical solutions cluster together after 1 s, showing that a pseudo-steady-state condition is reached fairly early. The pseudo-steady-state assumption is a valid one, especially in the beginning, when R_{bnd} is very far away and the colonies are very small and do not interact with each other. Note that the change in concentration is in the micromolar range when the colony radius is 1.6 μm, while at a radius of 20 μm, the change is about 1,000-fold, in the millimolar range. This difference between the orders of magnitude of the concentration changes is more important than the difference between the two calculation methods when the colonies are small. At these concentrations, there will be no significant change in the pH (equation 4).

The main difference between the steady-state analytical solution and the numerical solution can be attributed to the application of the boundary conditions at the center of the colony (R_0) and at R_{bnd}. In the analytical solution, using boundary conditions (equations 9 and 16) yields solutions where the integration constant a (Table 4) equals zero and the concentration of lactic acid at infinity also equals zero. The second condition, where the concentration of lactic acid at infinity is equal to zero, implies that there is no interaction between the colonies.

We cannot use the pseudo-steady-state solution for the

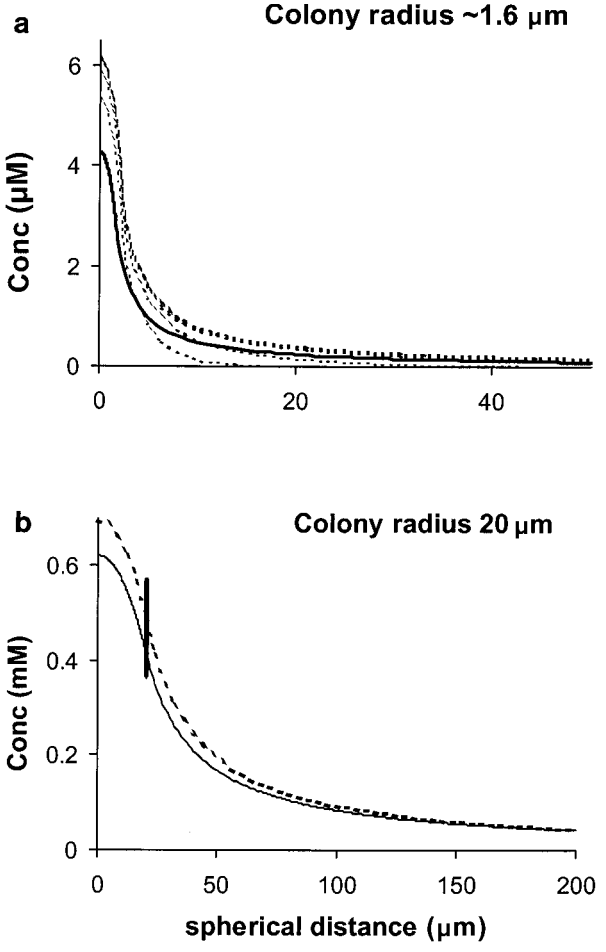

FIG. 3. Comparisons of concentration (Conc) profiles produced by using the pseudo-steady-state analytical (——) and numerical (------) solutions for colonies with radii of 1.6 and 20 μm. In panel a, the curves of the numerical solutions indicate concentration gradients at 0.1, 1, 60, 1,800, and 2,400 s. Here the colony consists of two cells (packing density, 10^{17} cell/m^3) after a doubling time of ~40 min. The order of magnitude of the concentration of lactic acid is micromolar in panel a and millimolar in panel b. The bar in panel b indicates the colony radius.

whole growth period because it is only valid until a certain radial distance from the center of a colony (200 μm). However, the steady-state solution is still very valuable for the initial, most relevant, stages of the growth process. It can tell us when interactions start to become important in colonies and whether this is significantly different from broth cultures. Interaction studies of homogeneous broth cultures of *L. curvatus* have already been carried out (6). They also defined the onset of interaction as 10% deviation of the specific growth rate from its maximum initial value.

Model predictions. Figures 4 and 5 show comparisons of the growth of *L. curvatus* in broth and that in agar for three different inoculation densities (1, 10, and 100 CFU/ml) at initial pHs of 6 and 7. A trend can be seen in Fig. 4 and 5. As the inoculation density is decreased, growth in the agar samples starts to deviate from growth in the broth samples. This deviation is greatest in the sample with an inoculation density of ~1 CFU/ml.

The model was able to describe these trends very well. It should be noted that these are not fits but predictions based on parameters from the literature and from earlier experiments (6, 8, 15). The lag time was the exception, as it was fitted by using data from the 100-CFU/ml experiments. There was very little difference between the analytical and numerical approaches for the first 20 h of growth. Since this period also covers the growth phase when deviations start to occur in the agar samples, the analytical approach is a very useful, computationally simple, tool for indicating when interactions become important. The numerical solutions were able to predict the growth kinetics until the end. Cells are just added to the edge of the colony as long as the conditions there are still favorable. In Fig. 5, the numerical solutions tended to overestimate the data points after 20 h.

One possible explanation could be the choice of the effective diffusion coefficient. We used a constant effective diffusion coefficient of $2.81 \cdot 10^{-10}$ m^2/s for the simulations. Decreasing the effective diffusion coefficient to $<10^{-10}$ m^2/s causes the numerical solution to underestimate the data points after 20 h (P. K. Malakar, unpublished data). The variability of effective diffusion coefficients in bacterial colonies has been reported previously (9).

Another interesting feature of Fig. 4 is the slight increase in the growth lag in solid medium (shifts to the right). The samples in the solid medium were inoculated at 35°C and left to cool down to 30°C before being placed into the 30°C incubator. This was because this was the minimum temperature at which the agar mixture remained molten enough for good mixing. The initial temperature shift may be the source of the lag. With inoculation at 50°C and cooling down to 30°C, the lag was even more pronounced (Malakar, unpublished).

Interactions. For all of the broth culture samples, the transition from the exponential phase to the stationary phase was quite sudden. This is because near the end of the exponential phase, many cells are present and a doubling of such a huge number of cells will only shorten the onset of the stationary phase. In the agar cultures, there is a more gradual transition from the exponential to the stationary phase in the samples with 10 and 1 CFU/ml. In these samples, the exponential phase ends much earlier due to the local accumulation of lactic acid in the colonies. The cells in the center of the colony start to slow down their growth, thus decreasing the volume-averaged specific growth rate, $\bar{\mu}_m$. The vertical lines in Fig. 4 show the predicted interaction times during which $\bar{\mu}_m$ is changed by 10% (analytical solution, Table 6). This should signal the end of the exponential phase in the solid medium while growth was still exponential in the broth culture.

Table 6 compares the interaction times (t_{int}) by using the various solutions of the coupled growth and diffusion equations. The concentrations of lactic acid at which growth is changed by 10% from its initial maximal rate are 8.5 and 16.8 mM for a medium at initial pHs of 6 and 7 (Appendix B). At these interaction concentrations of lactic acid, the pH have decreased to 5.7 and 6.1, respectively. The fourth column of Table 6 shows the estimated time of interaction obtained from equations 22 and 23. The utility of using equations 22 and 23 to estimate the interaction time is evident. These two equations give a prompt estimation of the interaction time, while the interaction times for the pseudo-steady-state analytical and

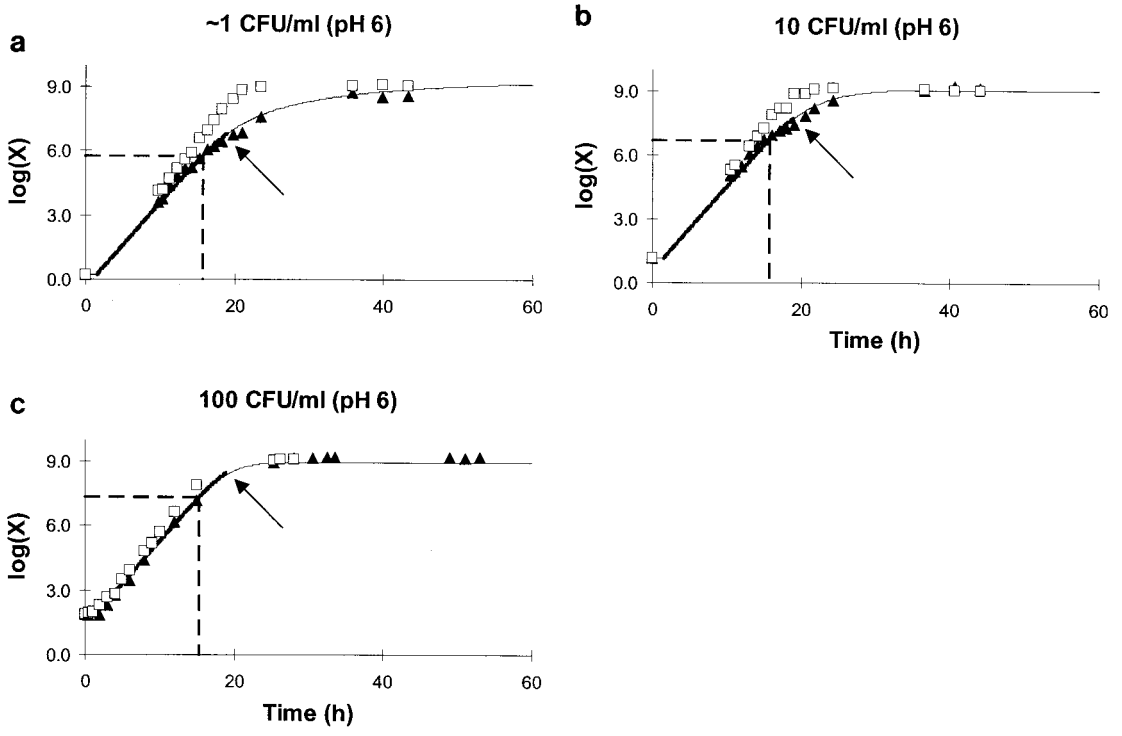

FIG. 4. Growth of *L. curvatus* at an initial pH of 6 in broth and in solid medium. Panels a, b, and c show growth data and predictions of the numerical and analytical solutions of growth in solid medium at initial inoculation densities of 1, 10, and 100 CFU/ml. The vertical and horizontal lines indicate the interaction time and interaction population density calculated by using equations 22 and 23. ▲, solid medium; □, broth; ——, numerical solution of growth in solid medium; ------, analytical solution of growth in solid medium. The arrow indicates the population density of a colony with a radius of 200 μm, which is the limit of the analytical solution. The analytical solution is superimposed over the numerical solution, indicating minor differences between the two methods.

numerical solutions (columns 5 and 6, Table 6) can only be estimated by running the associated Matlab programs.

On average, t_{int} obtained from equations 22 and 23 deviates by about 1% from the rest of the interaction times. Even though t_{int} obtained by the pseudo-steady-state and estimated solutions are derived from the same analytical equations, they differ because of the magnitude of the specific growth rate, μ_m. In equation 22, μ_m remains constant, while in the simulation to determine t_{int} by the pseudo-steady-state solution, μ_m is updated every 0.1 h. The analytical solutions also give good approximations of the interaction time compared to the numerical solutions because the colonies are still very small. At an initial pH of 6, the number of cells in the colony (analytical solutions) at the time of interaction is $\sim 6 \cdot 10^5$ and the radius of this colony is 113 μm. Similarly, the number of cells and the radius of the colony are $\sim 10^6$ and 134 μm at an initial pH of 7. The radius of these colonies is still much less than the 200-μm distance for which the pseudo-steady-state solutions are valid and thus also smaller than the R_{bnd} values (Table 5).

We can then distinguish two types of growth in the model solid system. In cultures where the initial inoculation density is relatively high (≥100 CFU/ml), growth approaches that of broth cultures. Here the colonies start to interact with each other earlier (intercolony interactions) as the total amount of lactic acid produced saturates the environment. Mass transfer limitation does not play an important role yet as the colony sizes are still quite small and diffusion is relatively fast.

At low inoculation densities, the total amount of acid produced takes a longer time to saturate the environment, thus allowing the colonies to grow bigger. Now mass transfer limitations do become important and there will be accumulation of lactic acid inside the colony. Here, the cells in a colony start to interact with each other earlier (intracolony interaction). This trend has also been shown with the aid of fluorescent ratio imaging techniques (7) when examining pH gradients in colonies of *L. curvatus*.

Conclusions. Mass transfer limitations can be significant in the development of bacterial colonies of *L. curvatus* in solid systems. However, these limitations start to play a role only when the colonies contain about 10^5 or more cells. At high initial inoculation densities (≥100 CFU/ml), growth in solid medium approaches growth in broth culture as the colonies do not grow to sizes at which mass transfer limitation becomes significant. One consequence of this research is that it is justified to use predictive models of broth culture studies for growth in solid medium when the initial inoculation concentration is high (≥100 CFU/ml). Also for low inoculation densities, predictions until growth of a factor of 10^5 are valid. These conditions cover most of the regimen relevant to food spoilage and certainly to food safety.

The derived analytical solutions are useful tools for quantifying the growth of microorganisms in solid medium. They can predict the onset of interaction quite well and are easy to use.

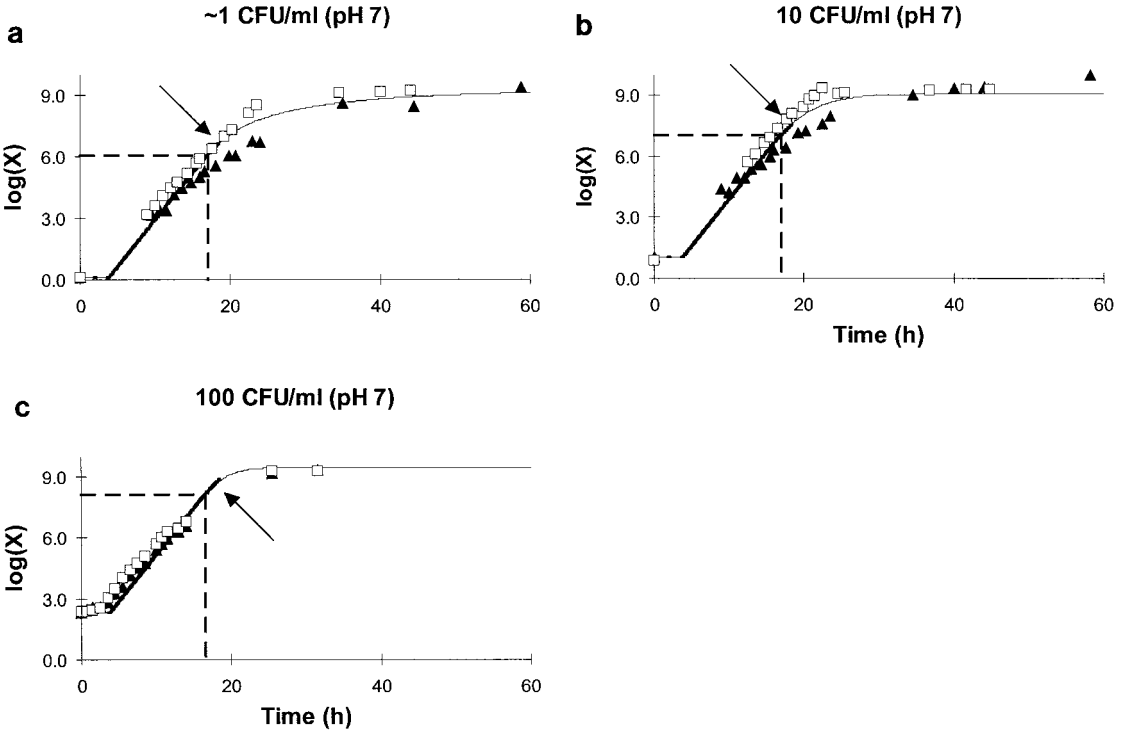

FIG. 5. Growth of *L. curvatus* at an initial pH of 7 in broth and in solid medium. Panel 5 a, b, and c show growth data and predictions of the numerical and analytical solutions of growth in solid medium at initial inoculation densities of 1, 10, and 100 CFU/ml. The vertical and horizontal lines indicate the interaction time and interaction population density calculated by using equations 22 and 23. ▲, solid medium; □, broth; ------, numerical solution of growth in solid medium; ——, analytical solution of growth in solid medium. The arrow indicates the population density of a colony with a radius of 200 μm, which is the limit of the analytical solution. The analytical solution is superimposed over the numerical solution, indicating minor differences between the two methods.

APPENDIX A

Applying the boundary condition at the center of a colony (equation 9) to the solution of equation 14 (Table 4) yields

$$\left(\frac{dP_i}{dr}\right)_{r=0} = -2\frac{q}{D_p}\frac{r}{6} + \frac{a}{r^2} = 0\ (r=0)\ (\text{mol/m}^3/\text{m}) \qquad \text{(A1)}$$

where the unknown a must be zero or else no solution is possible (division by zero). The resulting concentration profile inside the colony is

$$P_i = -\frac{q}{D_p}\frac{r^2}{6} + b\ (\text{mol/m}^3) \qquad \text{(A2)}$$

where b is an integration constant. The concentration outside the colony (Table 4) is given by

$$P_o = c - \frac{d}{r}\ (\text{mol/m}^3) \qquad \text{(A3)}$$

TABLE 6. Comparison of interaction times for growth in solid medium at initial pHs of 6 and 7

Initial pH	Lag, λ (h)	P_{int} (mol/m^3)[a]	t_{int} (h)		
			Estimated[b]	Analytical[c]	Numerical[d]
6	1.5	8.52	15.3	15.8	15.7
7	3.8	16.78	16.5	16.9	16.9

[a] P_{int} is the concentration of lactic acid in the colony when the specific growth rate is 90% of its initial value.
[b] Estimated by using equations 22 and 23.
[c] Derived with equations 17 and 18.
[d] Derived with equations 5 and 7.

where c and d are integration constants. At a very great distance from the colony, the concentration of lactic acid will still be zero (boundary condition at $r = \infty$, equation 16), resulting in

$$P_o(r=\infty) = c - 0 = 0\ (\text{mol/m}^3) \qquad \text{(A4)}$$

and thus

$$c = 0\ (\text{mol/m}^3) \qquad \text{(A5)}$$

At the colony radius, R_{col}, the fluxes

$$\left(\frac{dP_i}{dr}\right)_{r=R_{col}} = \left(\frac{dP_o}{dr}\right)_{r=R_{col}}\ (\text{mol/m}^3/\text{m}) \qquad \text{(A6)}$$

as they share a common boundary (equation 12), and this condition results in

$$-\frac{q}{3D_p}R_{col} = \frac{d}{R_{col}^2}\ (\text{mol/m}^4) \qquad \text{(A7)}$$

and

$$d = -\frac{q}{3D_p}R_{col}^3\ (\text{mol/m}^2) \qquad \text{(A8)}$$

Using condition 11 at R_{col} gives

$$-\frac{q}{D_p}\frac{R_{col}^2}{6} + b = \frac{q}{3D_p}R_{col}^2\ (\text{mol/m}^3) \qquad \text{(A9)}$$

and

$$b = \frac{q}{2D_p} R_{col}^2 \text{ (mol/m}^3\text{)} \quad \text{(A10)}$$

This completes the search for the values of the unknowns and yields

$$P_i = \frac{q}{2D_p}\left(R_{col}^2 - \frac{r^2}{3}\right) \; r \le R_{col} \text{ (mol/m}^3\text{)} \quad \text{(A11)}$$

and

$$P_o = \frac{1}{3}\frac{q}{D_p}\frac{R_{col}^3}{r} \; r > R_{col} \text{ (mol/m}^{-3}\text{)} \quad \text{(A12)}$$

which describes the concentration of lactic acid from the center of a colony until the distance R_{bnd}.

APPENDIX B

The volume-averaged concentration in the colony can be derived from

$$\bar{P} = \frac{\int_0^V P(r)dV}{V} \text{ (mol/m}^3\text{)} \quad \text{(B1)}$$

and

$$\bar{P} = \frac{3\int_0^{R_{col}} P(r)\, r^2 dr}{R_{col}^3} \text{ (mol/m}^3\text{)} \quad \text{(B2)}$$

and

$$\bar{P} = \frac{3\int_0^{R_{col}} \frac{q}{2D_P}\left(R_{col}^2 r^2 - \frac{r^4}{3}\right)dr}{R_{col}^3} \text{ (mol/m}^3\text{)} \quad \text{(B3)}$$

which equals

$$\bar{P} = \frac{3}{2D_p}\frac{q}{R_{col}^3}\left(R_{col}^2\frac{r^3}{3} - \frac{r^5}{15}\right)_0^{R_{col}} \text{ (mol/m}^3\text{)} \quad \text{(B4)}$$

resulting in

$$\bar{P} = \frac{2}{5}\frac{q}{D_p} R_{col}^2 \text{ (mol/m}^3\text{)} \quad \text{(B5)}$$

The concentration of lactic acid at the R_{col} is

$$P(r = R_{col}) = \frac{1}{3}\frac{q}{D_p} R_{col}^2 \text{ (mol/m}^3\text{)} \quad \text{(B6)}$$

and dividing this concentration by the volume-averaged concentration yields

$$\frac{\bar{P}}{P(r = R_{col})} = 1.2 \quad \text{(B7)}$$

Interactions occur when the specific growth rate, μ_{int}, is 90% of its initial value, μ_m, given by

$$\mu_{int} = 0.9\,\mu_m \text{ (1/s)} \quad \text{(B8)}$$

Since the substrate term on the right-hand side of equation 3 is close to unity and b_2P is negligible, the pH is the most important factor decreasing the specific growth rate. Substituting the initial pH (pH_0) and interaction pH (pH_{int}) into equation 3 results in

$$(pH_{int} - pH_{min})(pH_{max} - pH_{int}) = 0.9(pH_0 - pH_{min})(pH_{max} - pH_0) \quad \text{(B9)}$$

and solving for pH_{int} and substituting in equation 4 yields

$$P_{int} = \frac{pH_{int} - pH_0}{a_1 - a_2\, pH_{int}} \text{ (mol/m}^{-3}\text{)} \quad \text{(B10)}$$

Note that we assume that μ_{int}, pH_{int}, and P_{int} are approximately equal to their volume-averaged counterparts since the specific growth rate has only changed by 10%. If this is not assumed, then no analytical solution for the interaction time is possible.

APPENDIX C

The radius of a colony developing from one cell can derived from

$$V_{col} = \frac{N}{n} = \frac{4}{3}\,\pi\, R_{col}^3 \text{ (m}^3\text{)} \quad \text{(C1)}$$

with

$$N = \exp(\mu_m t) \text{ (cells)} \quad \text{(C2)}$$

resulting in

$$R_{col} = \sqrt[3]{\frac{0.75}{\pi}\frac{\exp(\mu_m t)}{n}} \text{ (meters)} \quad \text{(C3)}$$

where V_{col} is the volume; N is the number of cells in a colony, and n is the packing density.

APPENDIX D

The volume-averaged specific growth rate can be derived from

$$\frac{\int_0^v \mu_m(r)dV}{V} = \bar{\mu}_m \text{ (1/s)} \quad \text{(D1)}$$

with

$$V = \frac{4}{3}\pi r^3 \text{ (m}^3\text{)} \quad \text{(D2)}$$

and

$$dV = 4\pi r^2 dr \text{ (m}^3\text{)} \quad \text{(D3)}$$

which yields

$$\frac{3\int_0^{R_{col}} \mu_m(r) r^2 dr}{R_{col}^3} = \bar{\mu}_m \text{ (1/s)} \quad \text{(D4)}$$

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