Quantifying the effects of cell death and agar density on yeast colony biofilms using an extensional flow mathematical model

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Abstract: We used a combination of experiments, mathematical modelling, and parameter estimation to better understand how agar density affects colony biofilm growth of the yeast species *Saccharomyces cerevisiae*. We obtained 15 total experimental replicates on rectangular plates filled with 0.6%, 0.8%, 1.2%, and 2.0% agar. In the experiments, we measured the horizontal expansion over time, the proportion of living cells, and the colony biofilm aspect ratio, to quantify the colony biofilm size, composition, and shape, respectively. We modelled colony biofilm expansion using a thin-film extensional flow mathematical model. Fitting five unknown model parameters to mean experimental data revealed that nutrient uptake decreases and biofilm–substratum adhesion strength increases on harder agar. Sensitivity analysis, fitting to individual replicates, and synthetic data analysis confirmed that increased biofilm–substratum adhesion is the most consistent effect of harder agar. This finding aligns with similar results reported for bacteria, and suggests that substratum mechanics are important for yeast colony biofilm growth.

Keywords: Lubrication theory, thin-film approximation, viscous flow, multiphase flow, slip, *Saccharomyces cerevisiae*, parameter estimation

1 Introduction and Background

Biofilms are surface-dwelling communities of cells embedded within a viscous extracellular matrix (ECM) composed of extracellular polymeric substances (EPS) [1–3]. The bakers' yeast *Saccharomyces cerevisiae* is an important model organism in cell biology [4, 5], because of its ease of use in experiments and its similarity to higher eukaryotic cells [6]. Reynolds and Fink [7] developed an assay for *S. cerevisiae* biofilm formation, which requires the Flo11p glycoprotein known to promote cell-to-surface adhesion. Grown on agar media, the yeast forms a confluent mat that can fill a 90 mm Petri dish in under two weeks [8–10]. These single-species mats are less complex than naturally-occurring biofilms, but still involve the key feature of extracellular matrix deposition. We refer to a single-species mats as a *colony biofilm*. Due to similarities between yeast and the bacterium *Mycobacterium smegmatis* [11], Reynolds and Fink [7] hypothesised that the yeast colony biofilm expands by sliding motility. This term refers

to passive growth [12] where the cells and fluid expand as a unit, with low surface tension and weak adhesion to the substratum. Mathematical modelling, when combined with experimental observations, enables us to better understand these expansion mechanisms.

Mathematical modelling of colony biofilm growth is an established field [13, 14], encompassing agent-based [15–19], reaction–diffusion [20–28], and continuum mechanical [29–43] approaches. The earliest and most common models consider growth in a liquid culture medium from which the biomass obtains nutrients [44, 45]. In contrast, our yeast colony biofilms spread over an agar medium, bounded above by air. This scenario has been addressed previously by several authors [34–36, 46–50]. For *S. cerevisiae* specifically, Tam et al. [36] developed a thin-film extensional flow mathematical model for colony biofilm expansion by sliding motility [36]. However, Tam et al. [36] neglected cell death when comparing the model with experiments. In harsh environments, yeast cells will die by accidental cell death [51, 52], but can also undergo regulated cell death [22, 53] which releases nutrient for the benefit of the colony. Another limitation is that Tam et al. [36] only considered experiments on soft, low-density (0.3%) agar. Recent work [54, 55] has explored how agar density and cell–substratum adhesion impact growth in bacterial colonies [29, 54–56]. These works have revealed that hard, high-density substrates tend to reduce nutrient uptake [56], increase cell–substrate friction [55], promote wrinkle formation [29], and favour vertical over horizontal growth, increasing the biofilm–agar contact angle [55]. However, although we expect these effects of varying agar density to occur in *S. cerevisiae* colonies as well, their prevalence and their underlying mechanisms remain largely unexplored in fungal colonies.

We combine experiments and mathematical modelling to quantify the effects of cell death and agar density in *S. cerevisiae* yeast colony biofilms. In Section 2, we describe our rectangular experimental assay and the image processing procedure. These methods yielded a total of 15 experimental colony biofilms on 0.6%, 0.8%, 1.2%, and 2.0% agar. We obtained measurements for colony biofilm width over time, cell viability data at three spatial locations, and a measure of the aspect ratio. Like bacteria [55], in our experiments harder agar favoured vertical growth over horizontal growth. To interpret these new experimental results, we modify the thin-film extensional flow model of Tam et al. [36] to incorporate colony biofilm composition data and the impact of cell–substrate adhesion. We then perform a more detailed comparison with experiments than our previous work. Using numerical optimisation, we fit five unknown parameters to the experimental data, obtaining a set of optimal parameters for each agar density. Consistent with previous studies in bacteria [55, 56], increased agar density reduces nutrient uptake and increases cell–substratum friction. In contrast, biomass production rate, cell death rate, and nutrient consumption rate have less variability across agar densities. We confirm the robustness of these results using parameter sensitivity analysis and synthetic simulations. These analyses suggest that differences in cell–substratum adhesion are robust, whereas differences in nutrient uptake are less robust. Thus, we confirm that cell–substratum adhesion is a vital mechanism explaining differentiated growth of *S. cerevisiae* colony biofilms on varying density media.

2 Yeast Colony Biofilm Experiments

A total of 15 experimental yeast colonies were grown in rectangular Petri dishes, across agar densities $a \in \{0.6, 0.8, 1, 2, 2.0\}\%$. All replicates were stained using Phloxine B dye (10 µM), to indicate locations of cell death [57, 58]. For each replicate, we took eight photographs on Days $2, 4, 6, 8, 10, 12, 14$, and 21,

and used image processing to obtain the colony biofilm width. We also measured the proportions of living and dead cells at three spatial locations in the colony biofilm, averaged over two replicates (one each on 0.6% and 2.0% agar), on Day 14 of the experiments. Total cell counts were obtained on Day 21, which we used to estimate the aspect ratio. We introduce the following notation for experimental measurements, enabling comparison with the mathematical model:

- $S_i(t_j; a)$: Experimental colony biofilm half-width for replicate $i = 1, 2, 3, 4$ on agar density a, at time t_j, for $j = 1, \ldots, 8$, where t_j represents the time of the j-th photograph.
- $\Phi_k = \Phi(x_k)$: Cell volume fraction at spatial position x_k, for $k = 1, 2, 3$, averaged over all replicates and agar densities. The three measurements were taken at approximately $x_1 = 0$, $x_2 = S(t)/2$, and $x_3 = S(t)$, where $t = 14$ days.
- $A(a)$: Colony biofilm aspect ratio on agar density a, averaged over all replicates. The aspect ratio is defined as the colony biofilm's maximum vertical thickness divided by its mean half-width. We obtained one estimate per experiment, on Day 21.

When comparing the model with experiments, we ensure that quantities are dimensionless by scaling the experimental results according to the model nondimensionalisation introduced in Section 3.

2.1 Experimental Method

Σ1278b, a diploid prototrophic strain of *Saccharomyces cerevisiae*, was used in these experiments. Yeast Peptone Dextrose (YPD) medium ($10\,\mathrm{g\,L^{-1}}$ yeast extract, $20\,\mathrm{g\,L^{-1}}$ peptone, $20\,\mathrm{g\,L^{-1}}$ glucose) either as a liquid or with 0.6, 0.8, 1.2 and 2.0% agar for solid media was prepared by filter sterilising $2 \times$ YPD and mixing with an equal volume of molten 12, 16, 24 or $40\,\mathrm{g\,L^{-1}}$ (BD Bacto™ Agar, Becton, Dickinson and Company, New Jersey, USA). Phloxine B was also added to YPD agar at 10 µM. A 5 mL yeast culture was grown for 24 hours in liquid YPD prior to inoculation on plates. A straight streak of yeast culture was applied to YPD agar in a 100 mm square Petri dish with a 4.5 mm plastic inoculation loop guided along a suspended ruler edge. The initial cell density was $1.2 \times 10^8\,\mathrm{cells\,mL^{-1}}$ and the streak width approximately 2 mm. Plates were cling wrapped and incubated agar side down for up to 21 days at 25 °C. Macroscopic plate images were captured with an Apple iPhone 12 Pro. Cross sections that spanned the entire width of the colony biofilm streak (and 5 mm tall) were cut from plates with a scalpel and imaged using a Nikon Eclipse 50i microscope and Nikon camera (DS-2MBW, Nikon, Japan). Multiple images were taken across each section and stitched together with Fiji software (ImageJ).

The proportion of stained cells from samples from plates was measured by flow cytometry with a Guava 12 HT system and Guava EasyCyte™ software (Luminex, Yokohama, Japan; formerly Millipore, Darmstadt, Germany). The flow cytometry was equipped with Violet (405 nm), Blue (488 nm, 150 mW), Red (642 nm) excitation lasers, along with 450/45 nm and 664/20 nm emission filters. Cell samples were collected with sterile pipette tips or washed from 2 or $4\,\mathrm{cm}^2$ agar slices spanning the entire width of the area containing yeast and diluted in 5 mL phosphate buffered saline. Cells fluorescent due to the uptake of Phloxine B were considered non-viable. In parallel, the total number of cells was also enumerated using a hemocytometer and a Nikon Eclipse 50i microscope.

2.2 Image Processing and Quantification

We obtained a time series of photographs of the yeast colony biofilm for each experimental replicate. Example photographs from one replicate on 0.6% agar and one replicate on 2.0% agar are shown in Figure 2.1. To obtain the colony biofilm width in an experimental photograph, we used the known

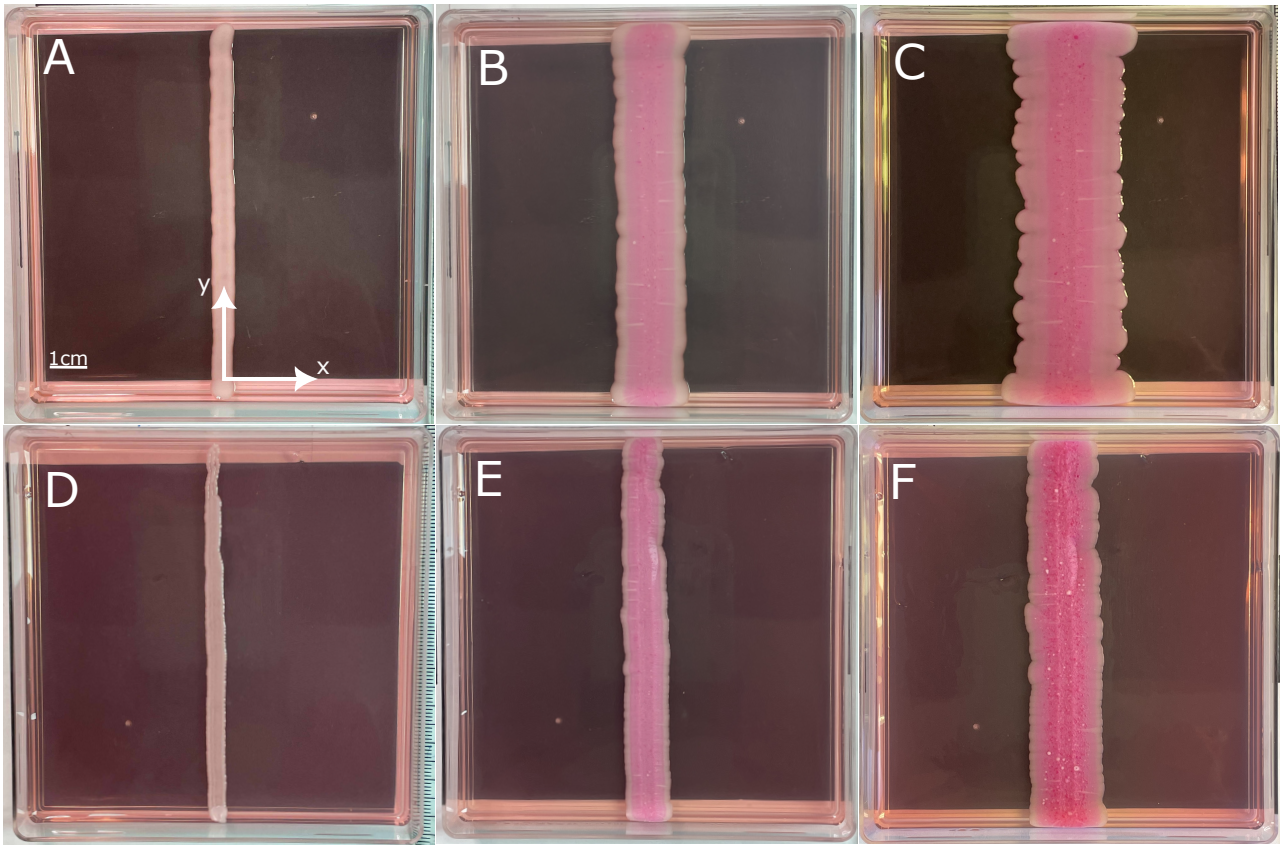

Figure 2.1: Experimental photographs of yeast colony biofilm growth over 21 days on 0.6% and 2.0% agar. Darker pink indicates regions of dead cells stained with Phloxine B. (A) Colony biofilm grown on 0.6% agar (Day 2), with axes and scale bar included. (B) Colony biofilm grown on 0.6% agar (Day 10). (C) Colony biofilm grown on 0.6% agar (Day 21). (D) Colony biofilm grown on 2.0% agar (Day 2). (E) Colony biofilm grown on 2.0% agar (Day 10). (F) Colony biofilm grown on 2.0% agar (Day 21).

Petri dish size of 100 mm to determine the physical distance corresponding to a pixel. After obtaining this scaling factor, we manually rotated and cropped each photograph to remove the Petri dish from the image. We then used Julia's `FileIO` package and `Images` library to import the photograph and apply Otsu's method to obtain a binary image. We then computed the total number of visible pixels in the colony biofilm, divided by the number of pixels in the y-direction of the photograph, and applied the scaling factor, to determine an approximation for the colony biofilm width. The mean colony biofilm half-widths over time for all agar densities are shown in Figure 2.2.

Our experiments also provide measurements of the proportions of living and dead cells. Photographs of colonies stained with Phloxine B (Figure 2.1) suggest that the proportion of living cells is maximum at the leading edge, and decreases towards the centre of the colony biofilm. To investigate overall cell viability, we obtained one measurement from a colony biofilm on soft (0.6%) agar, and one measurement from a colony biofilm on hard (2.0%) agar. Both measurements were taken on Day 14. In sampling

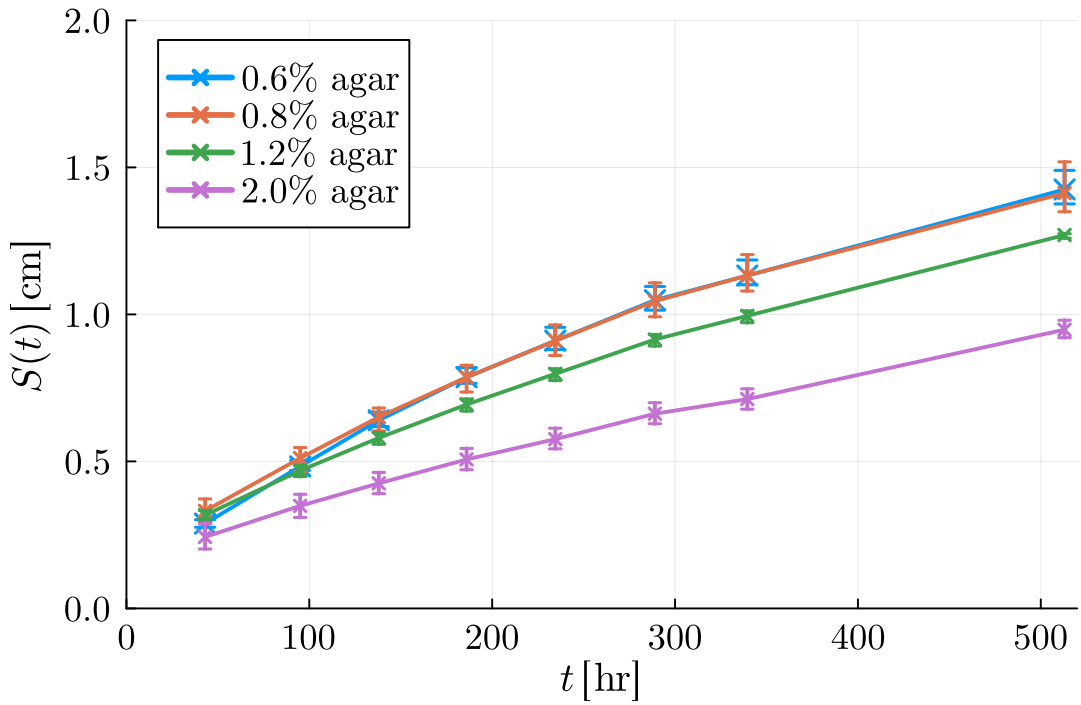

Figure 2.2: Mean experimental half-width, $S(t)$ for colony biofilms grown on different density agar media, plotted against time. Error bars indicate the range observed across all experiments ($n = 4$ for all agar densities, except $n = 3$ for 2.0% agar).

from a $2\,\mathrm{cm}^2$ region of agar across the full width of the colony biofilm, we found 89.5% total cell viability on soft (0.6%) agar, and 89.9% on hard (2.0%) agar. Since there was negligible difference in overall viability between agar densities, we assumed that each colony biofilm has the same composition regardless of replicate and agar density. We then obtained smaller samples from the same two colonies at three localised regions of the colony biofilm, to quantify how cell viability depended on position. Averaging across both 0.6% and 2.0% agar, we obtained the results shown in Table 2.1. In previous experiments, some colony biofilms stained with Phloxine B included a red ring of elevated cell death near the rim, which could be due to regulated cell death [22]. However, we did not observe an analogous region in these experiments. Instead, the proportion of dead cells is highest in the centre of the colony biofilm, and decreases towards the leading edge (Table 2.1). This observation suggests that cell death is predominantly due to accidental cell death in these colony biofilms [22].

Table 2.1: Average colony biofilm composition (proportion of living cells) at three regions along the colony biofilm. Proportions averaged from one replicate each on 0.6% and 2.0% agar, on Day 14.

Centre ($x_1 = 0$)	**Middle** ($x_2 = S(t)/2$)	**Edge** ($x_3 = S(t)$)
0.855	0.874	0.918

We used cross-sectional microscopy and cell count data to estimate the colony biofilm thickness. One limitation of our experimental method is that measuring the vertical profile was only possible after cutting the colony biofilm and imaging the cross-section. During cutting, the biofilm–substratum and

biofilm–air interfaces of colonies grown on soft agar changed shape significantly. Consequently, we could not obtain reliable thickness measurements for colony biofilms grown on soft agar. Given these experimental challenges, we only measured the physical aspect ratio from micrographs of colony biofilm cross-sections on hard (2.0%) agar, such as Figure 2.3. This yielded the aspect ratio of $A(2.0) = 0.0683$ for 2.0% agar.

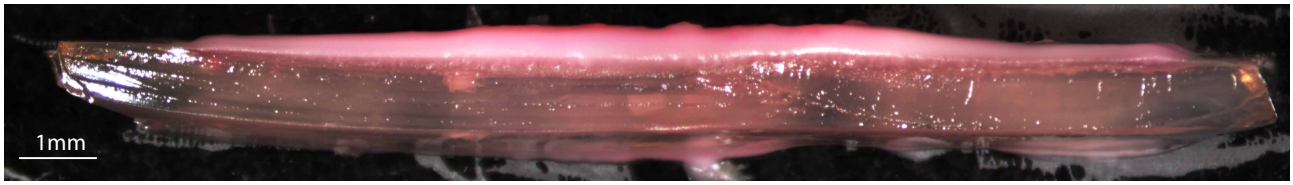

Figure 2.3: Cross-section of a colony biofilm grown on 2.0% agar, after 21 days of growth. We used this micrograph to measure the physical aspect ratio for 2.0% agar. We subsequently estimated the aspect ratio for 0.6%, 0.8%, and 1.2% agar using cell count data.

We used total cell counts to estimate the aspect ratios for 0.6%, 0.8%, and 1.2% agar. Cell count data were collected using a hemocytometer and a Nikon Eclipse 50i microscope on Day 21, providing an accurate measure of biomass volume. For each experiment, we used a 1 cm strip to count the mean number of cells per mm in the y-direction of the dish. The ratio of cell count to mean width then provided an estimate for the colony biofilm thickness, in cells per unit area in the (x, y) plane. Provided the cell sizes and colony biofilm compositions are similar for each agar density, the ratio of thickness in cells mm^{-2} to half-width in mm will be approximately proportional to the physical aspect ratio. Consequently, we estimated the physical aspect ratio by scaling the cell count aspect ratio, by a factor chosen to ensure that the aspect ratio for 2.0% agar is the measured value, $A(2.0) = 0.0683$. Table 2.2 shows these calculations, and the quantitative trends are consistent with the observations that colonies are thicker on harder agar than on softer agar.

Table 2.2: Aspect ratio calculations based on the experimental cell count data from Day 21 on agar media of different density.

Agar Density, a (%)	n	**Mean Count** (cells mm^{-1})	**Mean Half-Width** (mm)	**Mean Thickness** (cells mm^{-2})	**Aspect Ratio,** $A(a)$
0.6	4	1.06×10^8	14.3	7.44×10^7	0.0306
0.8	4	1.40×10^8	14.1	9.91×10^7	0.0411
1.2	4	1.60×10^8	12.7	1.25×10^8	0.0577
2.0	3	1.12×10^8	9.5	1.14×10^8	0.0683

3 Mathematical Model

We develop a thin-film model for colony biofilm growth, similar to those used by several authors [31, 34–36]. The base model considers colony biofilm growth along and perpendicular to a solid substratum of width X_s and depth H_s. The colony biofilm exists in the region $\Omega_b(t)$ bounded by $0 < x < S(t)$, and $0 < z < h(x, t)$, where $S(t)$ is the colony biofilm half-width, and $z = h(x, t)$ is the biofilm–air

interface, which is a free surface. We model cellular material and the EPS matrix as viscous fluids [59]. We partition the biomass into two phases, a living phase that contributes to growth and nutrient consumption, and an inactive phase of passive fluid. To incorporate the two phases into the model, we introduce the volume fractions of the living phase, $\phi_n(x, z, t)$, and the inactive phase, $\phi_m(x, z, t)$. In defining these volume fractions, we assume that an averaging process has occurred, as is standard in multiphase models [60]. The living biomass accounts for proliferating cells, and the inactive biomass phase represents dead cells. Since the proportion of cells to ECM in our experiments is unknown, we assume that both phases also contain extracellular fluid, such that the amount of ECM in each phase is proportional to the volume of cellular material. The no-voids assumption, $\phi_n + \phi_m = 1$, applies throughout the domain.

The colony biofilm expands by cell proliferation and ECM production, driven by the consumption of nutrients supplied from the substratum. We assume that the nutrients occupy no volume, and represent them as concentrations. We denote the nutrient concentration in the substratum as $g_s(x, z, t)$. This variable is defined in the substratum domain Ω_s, which is the fixed region bounded by $0 < x < X_s$ and $-H_s < z < 0$. During growth, the colony biofilm takes up nutrients from the substratum through the biofilm–substratum interface. We denote the nutrient concentration in the colony biofilm as $g_b(x, z, t)$, defined in $\Omega_b(t)$. A schematic illustrating the model geometry is shown in Figure 3.1.

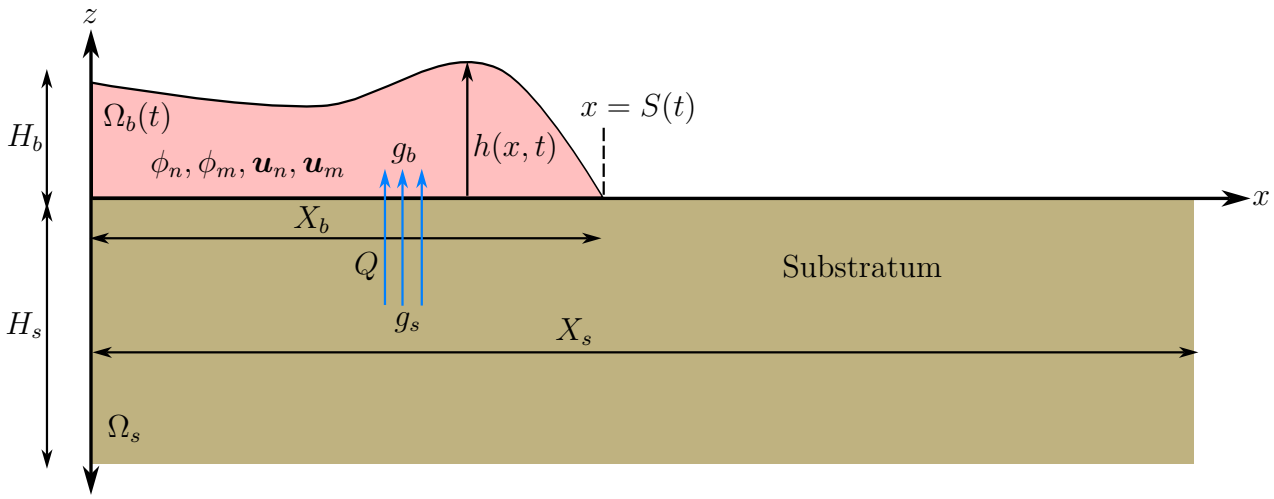

Figure 3.1: Schematic of the colony biofilm and agar geometry, showing the mathematical model variables. See the main text for the definitions of each symbol.

3.1 Governing Equations and Boundary Conditions

The governing equations arise from from mass and momentum conservation. Their derivation closely follows Tam et al. [36], and we summarise the key assumptions here. Cells catabolise glucose to facilitate cell proliferation, producing new biomass [61]. We adopt first-order kinetics to represent these processes. We assume that cell death occurs at a rate proportional to the volume fraction of living cells, which represents random accidental cell death. On death, we assume that cells immediately form part of the inactive phase, without any corresponding change in volume [31]. This assumption is consistent with the hypothesis that cell death influences the ECM composition [52]. We assume that nutrients disperse by diffusion in the substratum, and by diffusion and advection with both fluid phases in the colony biofilm, where they become available for consumption. Since yeast colony biofilms expand as a

unit [12], we assume that there is large interphase drag [62–64], such that both fluid phases move with the common velocity $\boldsymbol{u}_n = \boldsymbol{u}_m = \boldsymbol{u} = (u, w)$.

On applying the model assumptions, we obtain mass balance equations for the volume fractions and nutrient concentrations. These are

$$\phi_n + \phi_m = 1 \qquad \text{in } \Omega_b(t), \tag{3.1a}$$

$$\frac{\partial \phi_n}{\partial t} + \nabla \cdot (\phi_n \boldsymbol{u}) = \psi_n \phi_n g_b - \psi_d \phi_n \qquad \text{in } \Omega_b(t), \tag{3.1b}$$

$$\frac{\partial \phi_m}{\partial t} + \nabla \cdot (\phi_m \boldsymbol{u}) = \psi_d \phi_n \qquad \text{in } \Omega_b(t), \tag{3.1c}$$

$$\frac{\partial g_s}{\partial t} = D_s \nabla^2 g_s \qquad \text{in } \Omega_s, \tag{3.1d}$$

$$\frac{\partial g_b}{\partial t} + \nabla \cdot (g_b \boldsymbol{u}) = D_b \nabla^2 g_b - \eta \phi_n g_b \qquad \text{in } \Omega_b(t), \tag{3.1e}$$

where $\nabla = (\partial_x, \partial_z)$, the parameter ψ_n is the biomass production rate, ψ_d is the cell death rate, D_s and D_b are the diffusivities of nutrients in the substratum and colony biofilm, respectively, and η is the nutrient consumption rate. To obtain the momentum balance equations, we assume that both phases are incompressible viscous Newtonian fluids [36] with a common pressure p and dynamic viscosity μ. Conservation of momentum for both phases then yields

$$\nabla \cdot (\phi_\alpha \boldsymbol{\sigma}) + \boldsymbol{F}_\alpha = \mathbf{o} \quad \text{in } \Omega_b(t), \tag{3.2}$$

where

$$\boldsymbol{\sigma} = -\left(p + \frac{2\mu}{3} \nabla \cdot \boldsymbol{u}\right) \boldsymbol{I} + \mu \left[\nabla \boldsymbol{u} + (\nabla \boldsymbol{u})^T\right], \tag{3.3}$$

is the Cauchy stress tensor, $\boldsymbol{F}_\alpha$ represents external sources of momentum for phase $\alpha = n, m$, and $\boldsymbol{I}$ is the identity tensor. For both fluid phases we neglect an explicit contribution to the pressure due to growth, instead assuming that material incompressibility drives expansion in response to cell proliferation [31, 36]. Since the velocity field is in general not divergence free, we include a bulk viscosity term in (3.3), with the coefficient $-2\mu/3$ in accordance with Stokes' hypothesis [31, 36, 65, 66]. The momentum sources, $\boldsymbol{F}_\alpha$, consist of interphase drag and interfacial forces [36, 67–69]. These momentum sources are given explicitly by $\boldsymbol{F}_\alpha = -k(\boldsymbol{u}_n - \boldsymbol{u}_m) + p_\alpha \nabla \phi_\alpha$ for $\alpha = n, m$, where $k(\phi_n, \phi_m)$ is the interphase drag coefficient. Since we have assumed $\boldsymbol{u}_n = \boldsymbol{u}_m$, the drag component subsequently vanishes [62].

The boundary conditions include symmetry conditions for ϕ_n, ϕ_m, and u at $x = 0$, and no-flux conditions for the nutrient concentrations at the agar boundaries and the biofilm–air interface. We obtain another boundary condition by assuming that nutrients enter the colony biofilm at a rate proportional to the local concentration difference across the biofilm–substratum interface, neglecting osmotic effects. This yields the conditions

$$D_s \frac{\partial g_s}{\partial z} = D_b \frac{\partial g_b}{\partial z} = -Q\,(g_s - g_b) \qquad \text{on} \qquad z = 0, \tag{3.4}$$

where Q is a mass transfer coefficient describing the rate of nutrient uptake. We also apply standard boundary conditions from fluid mechanics, including no penetration on the biofilm–agar interface, and

the kinematic condition and tangential and normal stress conditions on the biofilm–air free surface. Since sliding motility involves reduced surface tension [12], we neglect surface tension in the normal stress condition on the free surface. Surface tension in colony biofilms might represent the strength of cell–cell adhesion on the free surface [70]. In the work of Tam et al. [36], surface tension had minimal impact on horizontal expansion. The surface tension coefficient does affect the colony biofilm shape, and can inhibit ridge formation on the colony biofilm surface. Our experimental results do not contain ridges, and have similar profiles to the solutions of Tam et al. [36] with zero surface tension. Hence, we neglect surface tension in this work.

For the tangential stress balance on the biofilm–substratum interface, we impose a general slip condition [69, 71], leading to

$$\mu \frac{\partial u}{\partial z} = \lambda u \quad \text{on} \quad z = 0, \tag{3.5}$$

where λ is a slip parameter representing the strength of biofilm–substratum adhesion. Whilst the general tangential stress condition (3.5) is was included in the models of Tam et al. [35] and Tam et al. [36], those works focused on the weak adhesion and perfect slip ($\lambda = 0$), and strong adhesion and no slip ($\lambda \to \infty$) scenarios, respectively. In this work, we consider the generalised case in which λ varies between these two limits, and estimate the value of λ using experimental data. The full dimensional model then consists of the governing equations (where all PDEs apply in $\Omega_b(t)$, except for (3.6c) which applies in Ω_s)

$$\frac{\partial u}{\partial x} + \frac{\partial w}{\partial z} = \psi_n \phi_n g_b, \tag{3.6a}$$

$$\frac{\partial \phi_n}{\partial t} + \frac{\partial}{\partial x}(\phi_n u) + \frac{\partial}{\partial z}(\phi_n w) = \psi_n \phi_n g_b - \psi_d \phi_n, \tag{3.6b}$$

$$\frac{\partial g_s}{\partial t} = D_s \left(\frac{\partial^2 g_s}{\partial x^2} + \frac{\partial^2 g_s}{\partial z^2} \right), \tag{3.6c}$$

$$\frac{\partial g_b}{\partial t} + \frac{\partial}{\partial x}(g_b u) + \frac{\partial}{\partial z}(g_b w) = D_b \left(\frac{\partial^2 g_b}{\partial x^2} + \frac{\partial^2 g_b}{\partial z^2} \right) - \eta \phi_n g_b, \tag{3.6d}$$

$$-\frac{\partial p}{\partial x} + \mu \left(\frac{\partial^2 u}{\partial x^2} + \frac{\partial^2 u}{\partial z^2} \right) + \frac{\mu}{3} \frac{\partial}{\partial x} \left(\frac{\partial u}{\partial x} + \frac{\partial w}{\partial z} \right) = 0, \tag{3.6e}$$

$$-\frac{\partial p}{\partial z} + \mu \left(\frac{\partial^2 w}{\partial x^2} + \frac{\partial^2 w}{\partial z^2} \right) + \frac{\mu}{3} \frac{\partial}{\partial z} \left(\frac{\partial u}{\partial x} + \frac{\partial w}{\partial z} \right) = 0, \tag{3.6f}$$

the boundary conditions

$$\frac{\partial g_s}{\partial z} = 0 \quad \text{on} \quad z = -H_s, \tag{3.7a}$$

$$D_s \frac{\partial g_s}{\partial z} = -Q\left(g_s - g_b\right) \quad \text{on} \quad z = 0, \tag{3.7b}$$

$$D_b \frac{\partial g_b}{\partial z} = -Q\left(g_s - g_b\right) \quad \text{on} \quad z = 0, \tag{3.7c}$$

$$w = 0 \quad \text{on} \quad z = 0, \tag{3.7d}$$

$$\mu \frac{\partial u}{\partial z} = \lambda u \quad \text{on} \quad z = 0, \tag{3.7e}$$

$$g_b \left(u \frac{\partial h}{\partial x} - w\right) = D_b \left(\frac{\partial g_b}{\partial x}\frac{\partial h}{\partial x} - \frac{\partial g_b}{\partial z}\right) \quad \text{on} \quad z = h, \tag{3.7f}$$

$$\frac{\partial h}{\partial t} + u \frac{\partial h}{\partial x} = w \quad \text{on} \quad z = h, \tag{3.7g}$$

$$-2\frac{\partial h}{\partial x}\left(\frac{\partial u}{\partial x} - \frac{\partial w}{\partial z}\right) + \frac{\partial u}{\partial z} + \frac{\partial w}{\partial x} - \left(\frac{\partial h}{\partial x}\right)^2 \left(\frac{\partial u}{\partial z} + \frac{\partial w}{\partial x}\right) = 0 \quad \text{on} \quad z = h, \tag{3.7h}$$

$$\begin{aligned} p + \frac{2\mu}{3}\left(\frac{\partial u}{\partial x} + \frac{\partial w}{\partial z}\right) \\ = \mu \left[2\left(\frac{\partial h}{\partial x}\right)^2 \frac{\partial u}{\partial x} + 2\frac{\partial w}{\partial z} - \frac{\partial h}{\partial x}\left(\frac{\partial u}{\partial z} + \frac{\partial w}{\partial x}\right)\right] \left[1 + \left(\frac{\partial h}{\partial x}\right)^2\right]^{-3/2} \end{aligned} \quad \text{on} \quad z = h, \tag{3.7i}$$

and initial conditions which we will specify later.

3.2 Nondimensionalisation

After establishing the mass and momentum balances, we simplify the governing equations using the thin-film approximation. We assume that the aspect ratios of the colony biofilm and agar substratum are equal, and exploit their thin geometries to write

$$\varepsilon = \frac{H_s}{X_s} = \frac{H_b}{X_b} \ll 1, \tag{3.8}$$

where H_b and X_b are the characteristic thickness and characteristic half-width of the colony biofilm, respectively. The characteristic colony biofilm thickness is chosen to be $H_b = X_b H_s / X_s$, which is not necessarily the initial colony biofilm thickness. This assumption is valid because the aspect ratio of the substratum in the experiments, $\varepsilon = H_s/X_s = 0.06 \ll 1$, lies within the aspect ratio range of mature colony biofilms (between 0.032 and 0.068 depending on agar density).

We adopt a nondimensionalisation based on the scaled variables

$$\begin{gathered} t = \frac{X_b^2}{D_b}\hat{t}, \quad (x, z) = \left(X_b \hat{x}, \varepsilon X_b \hat{z}\right), \quad (u, w) = \left(\frac{D_b}{X_b}\hat{u}, \frac{\varepsilon D_b}{X_b}\hat{w}\right), \\ g_s = G\hat{g}_s, \quad g_b = G\hat{g}_b, \quad \text{and} \quad p = \frac{\mu D_b}{X_b^2}\hat{p}, \end{gathered} \tag{3.9}$$

where G is the initial nutrient concentration in the substratum. The dimensionless governing equations

are then (dropping hats on dimensionless quantities)

$$\frac{\partial u}{\partial x} + \frac{\partial w}{\partial z} = \Psi_n \phi_n g_b, \tag{3.10a}$$

$$\frac{\partial \phi_n}{\partial t} + \frac{\partial}{\partial x}(\phi_n u) + \frac{\partial}{\partial z}(\phi_n w) = \Psi_n \phi_n g_b - \Psi_d \phi_n, \tag{3.10b}$$

$$\frac{\partial g_s}{\partial t} = D\left(\frac{\partial^2 g_s}{\partial x^2} + \frac{1}{\varepsilon^2}\frac{\partial^2 g_s}{\partial z^2}\right), \tag{3.10c}$$

$$\frac{\partial g_b}{\partial t} + \frac{\partial}{\partial x}(g_b u) + \frac{\partial}{\partial z}(g_b w) = \frac{\partial^2 g_b}{\partial x^2} + \frac{1}{\varepsilon^2}\frac{\partial^2 g_b}{\partial z^2} - \Upsilon \phi_n g_b, \tag{3.10d}$$

$$-\frac{\partial p}{\partial x} + \frac{\partial^2 u}{\partial x^2} + \frac{1}{\varepsilon^2}\frac{\partial^2 u}{\partial z^2} + \frac{1}{3}\frac{\partial}{\partial x}\left(\frac{\partial u}{\partial x} + \frac{\partial w}{\partial z}\right) = 0, \tag{3.10e}$$

$$-\frac{\partial p}{\partial z} + \varepsilon^2\frac{\partial^2 w}{\partial x^2} + \frac{\partial^2 w}{\partial z^2} + \frac{1}{3}\frac{\partial}{\partial z}\left(\frac{\partial u}{\partial x} + \frac{\partial w}{\partial z}\right) = 0, \tag{3.10f}$$

and the dimensionless boundary conditions are

$$\frac{\partial g_s}{\partial z} = 0 \quad \text{on} \quad z = -1/\delta, \tag{3.11a}$$

$$\frac{\partial g_s}{\partial z} = -\frac{Q^\dagger}{D}(g_s - g_b) \quad \text{on} \quad z = 0, \tag{3.11b}$$

$$\frac{\partial g_b}{\partial z} = -Q^\dagger (g_s - g_b) \quad \text{on} \quad z = 0, \tag{3.11c}$$

$$w = 0 \quad \text{on} \quad z = 0, \tag{3.11d}$$

$$\frac{\partial u}{\partial z} = \lambda^\dagger u \quad \text{on} \quad z = 0, \tag{3.11e}$$

$$g_b\left(u\frac{\partial h}{\partial x} - w\right) = \frac{\partial g_b}{\partial x}\frac{\partial h}{\partial x} - \frac{1}{\varepsilon^2}\frac{\partial g_b}{\partial z} \quad \text{on} \quad z = h, \tag{3.11f}$$

$$\frac{\partial h}{\partial t} + u\frac{\partial h}{\partial x} = w \quad \text{on} \quad z = h, \tag{3.11g}$$

$$-2\frac{\partial h}{\partial x}\left(\frac{\partial u}{\partial x} - \frac{\partial w}{\partial z}\right) + \frac{1}{\varepsilon^2}\frac{\partial u}{\partial z} + \frac{\partial w}{\partial x} - \left(\frac{\partial h}{\partial x}\right)^2\left(\frac{\partial u}{\partial z} + \varepsilon^2\frac{\partial w}{\partial x}\right) = 0 \quad \text{on} \quad z = h, \tag{3.11h}$$

$$\begin{aligned} p + \frac{2}{3}\left(\frac{\partial u}{\partial x} + \frac{\partial w}{\partial z}\right) = \Bigg[2\varepsilon^2\left(\frac{\partial h}{\partial x}\right)^2\frac{\partial u}{\partial x} + 2\frac{\partial w}{\partial z} \\ -\frac{\partial h}{\partial x}\left(\frac{\partial u}{\partial z} + \varepsilon^2\frac{\partial w}{\partial x}\right)\Bigg]\left[1 + \varepsilon^2\left(\frac{\partial h}{\partial x}\right)^2\right]^{-3/2} \end{aligned} \quad \text{on} \quad z = h. \tag{3.11i}$$

The dimensionless model (3.10) and (3.11) contains seven dimensionless parameters,

$$\begin{aligned} \delta = \frac{X_b}{X_s}, \quad \Psi_n = \frac{G X_b^2 \psi_n}{D_b}, \quad \Psi_d = \frac{X_b^2 \psi_d}{D_b}, \quad D = \frac{D_s}{D_b}, \\ \Upsilon = \frac{X_b^2 \eta}{D_b}, \quad Q^\dagger = \frac{H_b Q}{D_b}, \quad \text{and} \quad \lambda^\dagger = \frac{H_b \lambda}{\mu}. \end{aligned} \tag{3.12}$$

The parameter δ is the ratio of the colony biofilm length scale to the Petri dish length scale. The parameters Ψ_n and Ψ_d are the dimensionless biomass production and cell death rates, respectively, D is the nutrient diffusivity ratio, and Υ is the nutrient consumption rate. The parameter $Q^\dagger$ is the nutrient uptake rate by the colony biofilm, and $\lambda^\dagger$ is the dimensionless biofilm–substratum adhesion strength. Since we expect these quantities to be small, we consider the distinguished limit in which $Q^\dagger$ and $\lambda^\dagger$ are

of $\mathcal{O}(\varepsilon^2)$ size as $\varepsilon \to 0$. Accordingly, we rescale $Q^\dagger$ and $\lambda^\dagger$ by setting $Q^\dagger \sim \varepsilon^2 Q^*$ and $\lambda^\dagger \sim \varepsilon^2 \lambda^*$, where Q^* and λ^* are $\mathcal{O}(1)$ quantities as $\varepsilon \to 0$. Nutrient uptake and slip then enter the leading-order model through $\mathcal{O}(\varepsilon^2)$ corrections to the boundary conditions. This regime represents weak biofilm–substratum adhesion, which is suitable for expansion by sliding motility [36]. Our parameter estimation will predict that Q^*, λ^*, and the other dimensionless quantities in (3.12) are of $\mathcal{O}(1)$ size, indicating that this scaling regime is suitable.

3.3 Extensional Flow Thin-Film Approximation

The scaling regime described in Section 3.2 leads to an extensional flow [31, 72]. The extensional flow regime produces more realistic colony biofilm shapes than the lubrication regime [35], which assumes larger pressure and surface tension. We apply the thin-film approximation by expanding variables in power series of ε^2,

$$h(x, y, t) \sim h_0(x, y, t) + \varepsilon^2 h_1(x, y, t) + \mathcal{O}(\varepsilon^4), \tag{3.13}$$

and so on. Applying expansions of the form (3.13) to the model (3.10) and (3.11) yields a problem where the nutrient concentrations and the horizontal component of the velocity are independent of z to leading order in ε. We also introduce the depth-averaged cell volume fraction,

$$\phi(x, t) = \frac{1}{h} \int_0^h \phi_n(x, z, t)\, \mathrm{d}z. \tag{3.14}$$

to integrate out the z-dependence in ϕ_n, such that all of the dependent variables are functions of x and t to leading order in ε. We also use the no-voids condition (3.1a) to eliminate ϕ_m from the system.

The spatially one-dimensional system to leading order in ε is (dropping the zero subscript on leading order quantities)

$$\frac{\partial h}{\partial t} + \frac{\partial}{\partial x}(uh) = \Psi_n \phi g_b h \qquad \text{on} \quad 0 < x < S(t), \tag{3.15a}$$

$$\frac{\partial}{\partial t}(h\phi) + \frac{\partial}{\partial x}(uh\phi) = \Psi_n \phi g_b h - \Psi_d \phi h \qquad \text{on} \quad 0 < x < S(t), \tag{3.15b}$$

$$\frac{\partial g_s}{\partial t} = D \frac{\partial^2 g_s}{\partial x^2} - \delta Q^* (g_s - g_b) H(S(t) - x) \qquad \text{on} \quad 0 < x < 1/\delta, \tag{3.15c}$$

$$h \frac{\partial g_b}{\partial t} + \frac{\partial}{\partial x}(uhg_b) = \frac{\partial}{\partial x}\left(h \frac{\partial g_b}{\partial x}\right) + Q^* (g_s - g_b) - \Upsilon \phi g_b h \qquad \text{on} \quad 0 < x < S(t), \tag{3.15d}$$

$$4 \frac{\partial}{\partial x}\left(h \frac{\partial u}{\partial x}\right) - \lambda^* u = 2\Psi_n \frac{\partial}{\partial x}(\phi g_b h) \qquad \text{on} \quad 0 < x < S(t), \tag{3.15e}$$

$$\frac{\mathrm{d}S}{\mathrm{d}t} = u(S(t), t), \tag{3.15f}$$

where $H(x)$ is the Heaviside step function. We include the Heaviside step function in (3.15c) because nutrient uptake only occurs where the colony biofilm is present. The parameter δ influences nutrient depletion from the substratum through Eqn. (3.15c), such that nutrients deplete more slowly on comparatively large substrates. The slip parameter λ^* provides a drag-like term in the leading-order momentum equation (3.15e), such that increasing λ^* represents stronger biofilm–substratum adhesion, inhibiting expansion.

To close the model, we apply the initial conditions

$$g_s(x,0) = 1, \quad g_b(x,0) = 0, \quad \phi(x,0) = 0, \quad h(x,0) = H_0\left(1 - x^2\right), \quad S(0) = 1, \tag{3.16}$$

and boundary conditions,

$$\begin{gathered} \left.\frac{\partial h}{\partial x}\right|_{(0,t)} = 0 \quad \left.\frac{\partial \phi}{\partial x}\right|_{(0,t)} = 0, \\ \left.\frac{\partial g_s}{\partial x}\right|_{(0,t)} = 0 \quad \left.\frac{\partial g_s}{\partial x}\right|_{(1/\delta,t)} = 0, \quad \left.\frac{\partial g_b}{\partial x}\right|_{(0,t)} = 0 \quad \left.\frac{\partial g_b}{\partial x}\right|_{(S(t),t)} = 0, \\ u(0,t) = 0, \quad \text{and} \quad \left.\frac{\partial u}{\partial x}\right|_{(S(t),t)} = \frac{1}{2}\Psi_n \phi(S(t),t) g_b(S(t),t). \end{gathered} \tag{3.17}$$

The condition for u at $S(t)$ arises by enforcing zero normal stress at $S(t)$. A complete derivation of the system (3.15)–(3.17) is available in the Supporting Material. An advantage of our two-phase moving-boundary model is that it enables comparison with new experimental data for the colony biofilm composition, expansion speed, and aspect ratio. We compute numerical solutions of the model (3.15)–(3.17), and compare the results with our experiments. We obtain numerical solutions to the mathematical model (3.15)–(3.17) using a front-fixing transformation and the Crank–Nicolson method, similar to Tam et al. [36]. Full details of the numerical scheme are available in the Supporting Material, and open source JULIA code is available on GitHub.

3.4 Model Parameters

Table 3.1 contains parameter values used throughout this work. The agar density, agar depth, amount of nutrient, duration, and Petri dish dimensions are known from the experiments. We also obtain estimates for the nutrient diffusivities from the literature, yielding estimates for D_s [73, 74] and D_b [75]. In the extensional flow regime, the colony biofilm expansion speed depends on the initial conditions [31, 36]. If the nutrient supply and the cell volume fraction are held constant, Ward and King [31] showed that the colony biofilm profile evolves to a travelling wave with speed proportional to $1/H_0$ as $t \to \infty$, where H_0 is the initial dimensionless colony biofilm thickness. Consequently, the dimensional parameter H_0 will impact expansion speed, and subsequently the comparison between the model and experiments. Although we have no reliable way to measure H_0 in the experiments, all experiments were inoculated in the same way. Therefore, we expect H_0 to be constant across all replicates, and to not vary with agar density. In the experiments, cells were applied to the agar using the tip of an inoculating loop of 4.5 mm diameter, such that $X_b \approx 2$ mm. The inoculum is a thin streak of cells and fluid that is rapidly absorbed into the agar layer. The cell density of this initial streak is much less than the cell density once the colony biofilm develops [76]. Consequently, we set $H_0 = 6\,\mu\text{m}$, which is an upper estimate for the minor axis diameter of a single cell of Σ1278b *S. cerevisiae* yeast [15, 77, 78].

We still require values for the biomass production rate Ψ_n, cell death rate Ψ_d, nutrient uptake coefficient Q^*, nutrient consumption rate Υ, and the slip coefficient λ^*. Since cellular behaviour and/or properties of the substratum determine these parameters, they are difficult to measure directly in experiments. We also hypothesise that these parameters might depend on the agar density. Therefore, we seek new estimates for the values of Ψ_n, Ψ_d, Q^*, Υ, and λ^* for each agar density, a. We cannot adopt the parameter values of Tam et al. [36], whose experiments were performed solely on 0.3% agar. Instead, we

Table 3.1: Estimated values for dimensional parameters in the mathematical model.

Parameter	**Symbol**	**Value(s)**	**Units**	**Source(s)**
Agar density (weight percentage)	a	$\{0.6, 0.8, 1.2, 2.0\}$	–	Experiment
Experimental duration	T	30780	min	Experiment
Petri dish half-width	X_s	50	mm	Experiment
Agar depth	H_s	3	mm	Experiment
Initial biofilm half-width	X_b	2	mm	Experiment
Initial biofilm thickness	H_0	0.006	mm	[15, 77, 78]
Initial nutrient concentration	G	1.5×10^{-4}	$\mathrm{g\,mm^{-2}}$	Experiment
Nutrient diffusivity (water)	D_0	4.04×10^{-2}	$\mathrm{mm^2\,min^{-1}}$	[73]
Nutrient diffusivity (substratum)	D_s	$(1-0.023a)D_0$	$\mathrm{mm^2\,min^{-1}}$	[74]
Nutrient diffusivity (biofilm)	D_b	$0.24D_0$	$\mathrm{mm^2\,min^{-1}}$	[75]

obtain parameter estimates by fitting the model to experimental data. Differences in these parameters between experiments on different agar densities will indicate how the agar density impacts colony biofilm growth.

4 Results and Discussion

We use numerical solutions and optimisation to fit five unknown parameters to experimental data on different density growth media. We obtain good fits to expansion speed, colony biofilm composition, and aspect ratio data. This approach reveals a stronger impact of nutrient limitation on softer agar, and increased biofilm–substratum adhesion on harder agar. A combination of sensitivity analysis and synthetic data suggests that the increase in biofilm–substratum adhesion is the most robust difference between the agar densities.

4.1 Parameter Estimation

We use a numerical optimisation method to obtain point estimates for the model parameters that correspond to experiments. Based on Table 3.1, the quantities X_b, G, D_b, ε, and μ are independent of the agar density. We seek one set of optimal parameters $\theta^*(a) = (\Psi_n(a), \Psi_d(a), Q^*(a), \Upsilon(a), \lambda^*(a))$ for each of the four agar densities used in our experiments. Our parameter estimates are based on minimising the difference between the experimental data and a numerical solution to the mathematical model. To perform the comparison, we introduce three summary statistics, $\mathcal{S}_S$, $\mathcal{S}_\Phi$, and $\mathcal{S}_A$. These statistics are the L^2-norms of the relative differences in half-width, composition, and aspect ratio, respectively, between the model solution with parameters θ and the experiments on agar density a.

Their definitions are

$$\mathcal{S}_S(\theta, a) = \sqrt{\sum_{j=1}^{8} \left[\frac{S(t_j;\theta) - \bar{S}(t_j, a)}{\bar{S}(t_j, a)} \right]^2}, \tag{4.1a}$$

$$\mathcal{S}_\Phi(\theta, a) = \sqrt{\sum_{k=1}^{3} \left[\frac{\phi_n(x_k, t^*;\theta) - \Phi_k}{\Phi_k} \right]^2}, \tag{4.1b}$$

$$\mathcal{S}_A(\theta, a) = \frac{1}{A(a)} \left| \frac{\max_x \varepsilon h(x, t^*;\theta)}{S(t^*)} - A(a) \right|, \tag{4.1c}$$

where

$$\bar{S}(t_j, a) = \frac{1}{n} \sum_{i=1}^{n} S_i(t_j; a), \tag{4.2}$$

is the half-width averaged over the n experimental replicates on agar density a, and t^* refers to the appropriate (dimensionless) time at which the experimental measurement was taken. Based on these summary statistics (4.1), we define the distance metric

$$\rho(\theta, a) = \sqrt{\mathcal{S}_S^2 + \mathcal{S}_\Phi^2 + \mathcal{S}_A^2}, \tag{4.3}$$

which provides a single measure of the difference between the model and experiments. Using the relative difference for each statistic (4.1) means that the half-width, composition, and aspect ratio will have similar weighting when calculating ρ. The optimal parameter values for agar density a are then

$$\theta^*(a) = \underset{\theta}{\operatorname{argmin}}\, \rho(\theta, a), \tag{4.4}$$

which is the set of parameters that minimises the distance between the model solution and experimental data.

To establish a procedure for computing the optimal parameter set $\theta^*(a)$ numerically, we use optimisation packages available in Julia. For a given agar density, the optimisation procedure is as follows:

1. We obtain a coarse estimate, $\theta_0^*(a)$, using the Differential Evolution method with adaptive weights [79, 80], as implemented in the global optimisation package `BlackBoxOptim.jl`. We terminate the black box optimisation after 100 function evaluations, where one function evaluation refers to a computation of $\rho(\theta, a)$, which involves solving the PDE model (3.15)–(3.17) for a set of parameters θ, and computing $\mathcal{S}_S$, $\mathcal{S}_\Phi$, and $\mathcal{S}_A$. The black box step provides a good initial guess for gradient-based optimisation. Increasing the number of function evaluations beyond 100 had negligible effect on the results.

2. Using $\theta_0^*(a)$ as the initial guess, we use the LBFGS [81–84] method in `Optim.jl` [85] to estimate $\theta^*(a)$. Where necessary, we approximate the gradient $\nabla_\theta \rho$ numerically using first-order finite differencing, and use the Hager–Zhang line search [86]. We apply box constraints to ensure that all parameters are positive, with 3 outer iterations, and absolute tolerances of $\varepsilon = 1 \times 10^{-6}$ for the objective function and the L^∞-norm of the minimiser.

Repeated application of the optimisation procedure to the same experimental data yielded only minor differences in the minimiser $\theta^*(a)$. Therefore, on each run our gradient-based optimisation method

converges effectively to a global minimum in ρ. Since each evaluation of the objective function $\rho(\theta, a)$ requires solving the model numerically once, the parameter estimation procedure is computationally expensive. In this work, we present all results using $N_\xi = 101$ grid points and $N_\tau = 401$ for consistency, as the computational cost for large-scale results becomes prohibitive for finer resolutions. Although the quantitative results at this resolution are grid-dependent, the qualitative conclusions reported herein remain valid as the numerical grid is refined. Further information regarding grid convergence is available in the Supporting Material. The optimal sets of dimensionless parameters are presented in Table 4.1. We obtained a good fit to experimental data for all agar densities considered. The accuracy of the fit is illustrated in Figure 4.1, where the colony biofilm size over time predicted numerically closely matches the experiments.

Table 4.1: Numerical solutions for the optimal set of dimensionless parameters, $\theta^*(a)$, for each agar density. The presented parameter values are the mean values from 5 repetitions of the optimisation routine, with the numerical solutions having been computed using $N_\xi = 101$ grid points and $N_\tau = 401$ time steps.

a	Ψ_n	Ψ_d	Q^*	Υ	λ^*	$\rho(\theta^*, a)$
0.6	0.301	0.00777	7.56	7.87	0.670	0.0437
0.8	0.365	0.00747	6.55	7.33	1.041	0.0536
1.2	0.409	0.00755	4.48	6.46	1.848	0.0636
2.0	0.302	0.00835	3.27	5.01	2.671	0.0625

The optimal biomass production and death rates, Ψ_n and Ψ_d, only vary slightly with changing agar density. These results reaffirm that proliferation and death rates are cell-associated properties with minimal dependence on the substratum. In contrast, Q^*, Υ, and λ^* vary more significantly with agar density. The nutrient uptake rate decreases with increasing agar hardness. He et al. [56] reported a similar result in *E. coli* bacteria. Since nutrient availability depends on both the uptake and consumption rate, the decrease in nutrient uptake is accompanied by a smaller decrease in nutrient consumption rate, Υ. The higher Q^* and Υ values indicate that nutrients deplete faster for colonies grown on softer agar. This depletion could also be due to agar dehydration or osmotic effects, which we neglect in the model. The most striking effect of increasing agar density is the increase in the dimensionless adhesion strength, λ^*. The small value of λ^* on 0.6% agar suggests that the perfect slip approximation ($\lambda^* = 0$) may be suitable for very soft agar. This supports the use of perfect slip by Tam et al. [36], whose experiments were on 0.3% media. The inclusion of non-zero λ^* becomes more important on harder agar. The increased λ^* on harder substrates increases drag-like friction, inhibiting horizontal expansion [87, 88] relative to vertical expansion. This explains the thicker colony biofilm profiles on harder agar. Similar behaviour in *E. coli* was also reported by He et al. [56], and this prediction aligns with the recent experimental results of Pokhrel et al. [55]. Given the potential for biological variability across experiments, we perform a series of analyses to assess the robustness of the results to changes in each parameter.

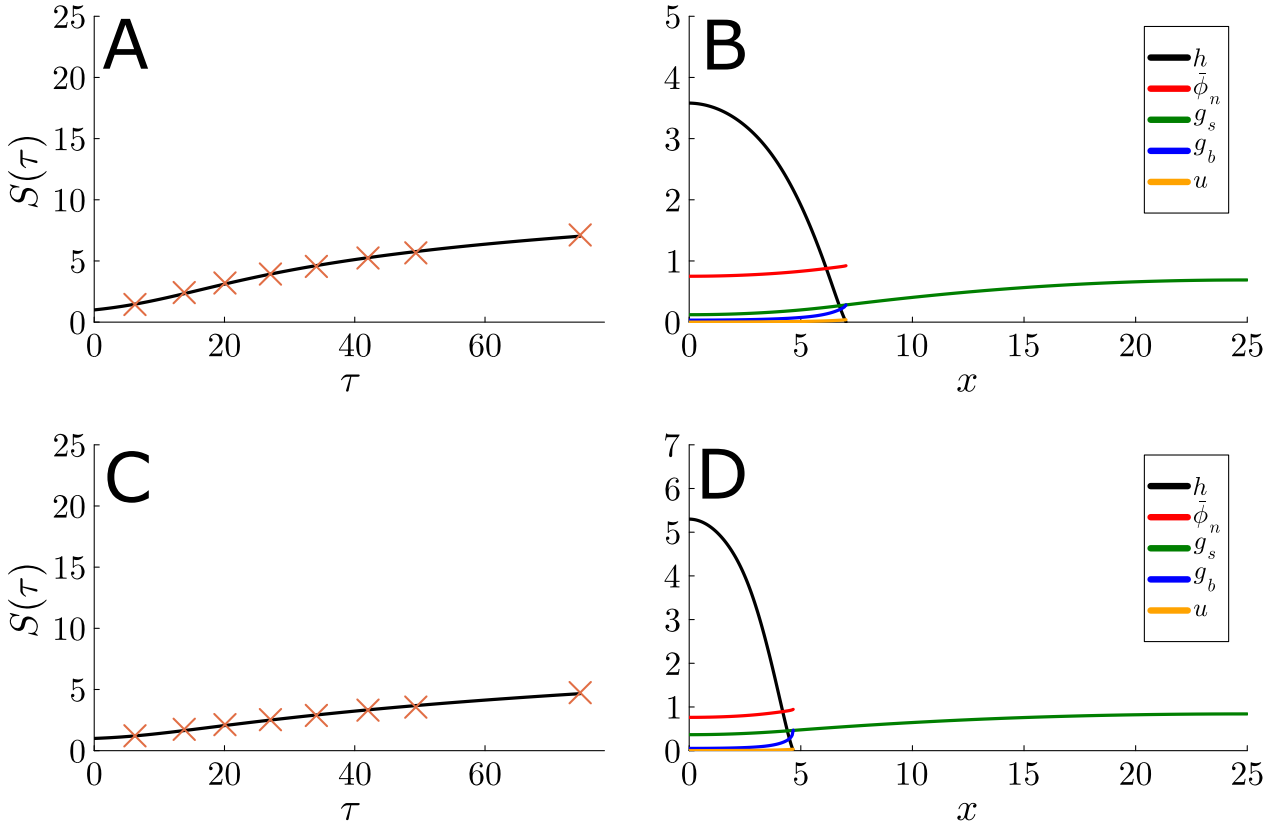

Figure 4.1: Numerical solutions to the mathematical model (3.15)–(3.17) with the estimated parameters from Table 4.1, for both soft (0.6%) and hard (2.0%) agar. The numerical solutions were computed using $N_\xi = 101$ grid points and $N_\tau = 401$ time steps. (A) Comparison between model solution (black curve) and experimental data (orange crosses) for the dimensionless colony biofilm size, $S(\tau)$, on 0.6% agar. (B) Numerical solution for $\tau = 74.6$, corresponding to Day 21 of the experiment, using optimal parameters for 0.6% agar. (C) Comparison between model solution (black curve) and experimental data (orange crosses) for the dimensionless colony biofilm size, $S(\tau)$, on 2.0% agar. (D) Numerical solution for $\tau = 74.6$, corresponding to Day 21 of the experiment, using optimal parameters for 2.0% agar.

4.2 Uncertainty and Sensitivity Analyses

Our experimental results consist of relatively few replicates that are subject to biological variation. We perform a series of analyses to determine the robustness of the parameter estimation results presented in Section 4.1. First, we investigate how varying the model parameters influences $\rho(\theta, a)$, the global distance metric that compares the mathematical model solution and experimental data. We present these results in the parameter-pair heat maps for 0.6% agar in Figure 4.2. In this figure, we vary parameters two at a time from zero to approximately three times their optimal values, holding the other parameters constant at their optimal values. The parameter-pair heat maps show the computed values of $\rho(\theta, a)$ as two parameters are varied.

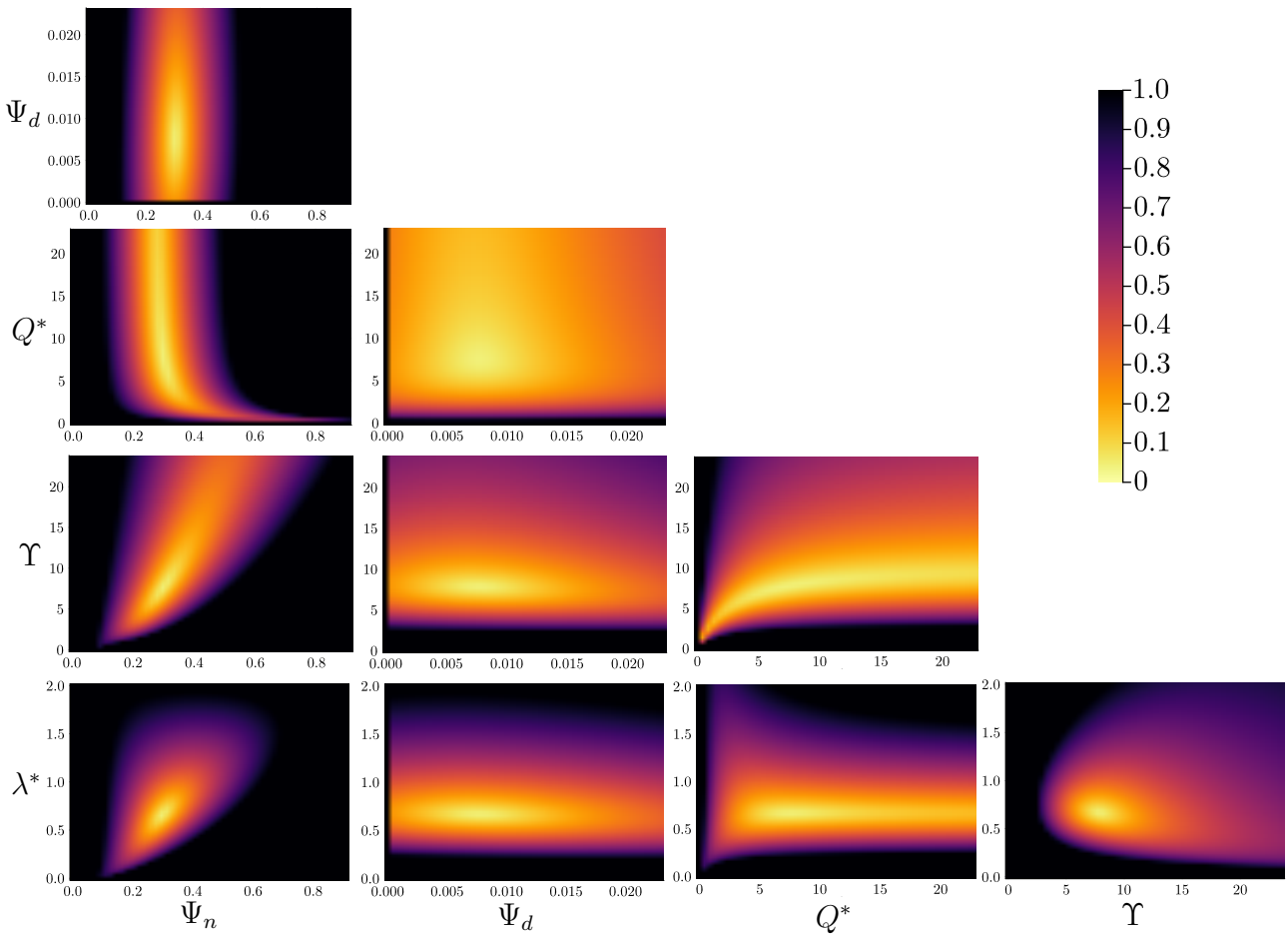

Figure 4.2: Parameter-pair heat maps for soft (0.6%) agar. Each plot shows the value of the global distance metric, $\rho(\theta, a)$, for a given set of parameters. Unless otherwise stated, all parameters take the optimal values listed in Table 4.1. When varied, each parameter ranges from zero to three times the optimal value (2,500 total simulations).

The biomass production rate, Ψ_n, has a narrow band of values that yield small values of $\rho(\theta, a)$. Increasing Ψ_n increases $S(t)$ in numerical solutions [36]. Varying Ψ_n from its optimal value causes large changes in ρ, suggesting that Ψ_n strongly influences the solutions and is well-identified by the experimental data. Compared to Ψ_n, varying Ψ_d has a smaller impact on the size of ρ. This is because cell death rate has a lesser effect on expansion speed [36], and only significantly impacts the living cell volume fraction, ϕ. Since colony biofilm composition had minimal variation with agar density in our experiments, the optimal value for Ψ_d remains similar across all agar densities considered.

Compared to other parameters, the distance $\rho(\theta, a)$ is also less sensitive to the nutrient uptake rate, Q^*. In the model, nutrients taken up from the substratum do not occupy volume, and therefore only contribute to growth when consumed. Nutrient consumption in the colony biofilm is influenced by both the uptake rate, Q^*, and consumption rate, Υ. Consequently, there is a range of pairs (Q^*, Υ) that similarly good fits to the data. Therefore, although our procedure identifies differences in Q^* and Υ across agar densities, the parameter Q^* is less well-identified than Ψ_n. A future version of the model that accounts for volumetric increase due to nutrient uptake, for example by osmotic swelling, could shed further light on the importance of nutrient uptake.

Increasing the biofilm–substratum adhesion strength λ^* increases the aspect ratio and decreases horizontal expansion [35]. Consequently, this parameter is well identified, with a comparatively narrower range of values yielding small $\rho(\theta, a)$. Since the aspect ratio increases with agar hardness, so too does the optimal adhesion strength λ^*. Taken together, this parameter-pair analysis reveals the key roles of the biomass production rate, nutrient consumption, and cell–substratum adhesion in determining the colony biofilm size and shape.

To investigate uncertainties in our parameter estimates further, we infer optimal parameter sets from individual experimental replicates, rather than from the mean of all experiments of a given agar density. These results are presented in Table 4.2, which reveal that the quantitative trends persist across different replicates of the same agar density.

Table 4.2: Optimal parameters for all individual experimental replicates.

a	Rep.	Ψ_n	Ψ_d	Q^*	Υ	λ^*	$\rho(\theta^*, a)$
0.6	1	0.299	0.00778	7.68	7.82	0.666	0.0446
	2	0.306	0.00778	6.84	8.01	0.691	0.0460
	3	0.312	0.00771	7.58	7.63	0.653	0.0448
	4	0.290	0.00780	8.10	8.02	0.674	0.0405
0.8	1	0.363	0.00762	5.94	6.35	0.973	0.0537
	2	0.312	0.00787	6.99	6.51	0.982	0.0451
	3	0.378	0.00731	6.84	8.27	1.107	0.0555
	4	0.416	0.00709	6.89	8.64	1.119	0.0590
1.2	1	0.380	0.00783	3.89	5.67	1.806	0.0629
	2	0.392	0.00766	4.50	6.09	1.785	0.0619
	3	0.429	0.00743	4.54	6.89	1.904	0.0652
	4	0.434	0.00731	5.13	7.21	1.883	0.0640
2.0	1	0.301	0.00835	3.26	5.01	2.681	0.0620
	2	0.236	0.00919	3.27	5.62	2.357	0.0510
	3	0.404	0.00757	3.59	7.37	3.088	0.0723

To supplement these results and account for experimental variability further, we repeated the optimisation procedure using synthetic data. For the synthetic aspect ratio data, we generated a synthetic cell count from a normal distribution with the mean and standard deviation from experiments, and

computed the corresponding aspect ratio following the method outlined in Section 2.2. To generate synthetic half-width data $S(t_j; a)$, we sampled once from the standard normal distribution, $z \sim \mathcal{N}(0, 1)$, and set $S(t_j; a) = \bar{S}(t_j; a) + z\sigma(t_j; a)$ where $\bar{S}(t_j, a) = (\sum_{i=1}^{n} S_i(t_j; a))/n$ are the mean measurements at each time point, and $\sigma(t_j; a)$ is the standard deviation. For the synthetic colony biofilm composition data, we scaled Φ_k by the same random number drawn from a uniform distribution $U(0.932, 1.05)$, which allows for composition results across the range observed in experiments. We generated 50 sets of synthetic data for each agar density, and obtained an optimal parameter set for each data set. The variability in these optimal parameters is shown in Figure 4.3.

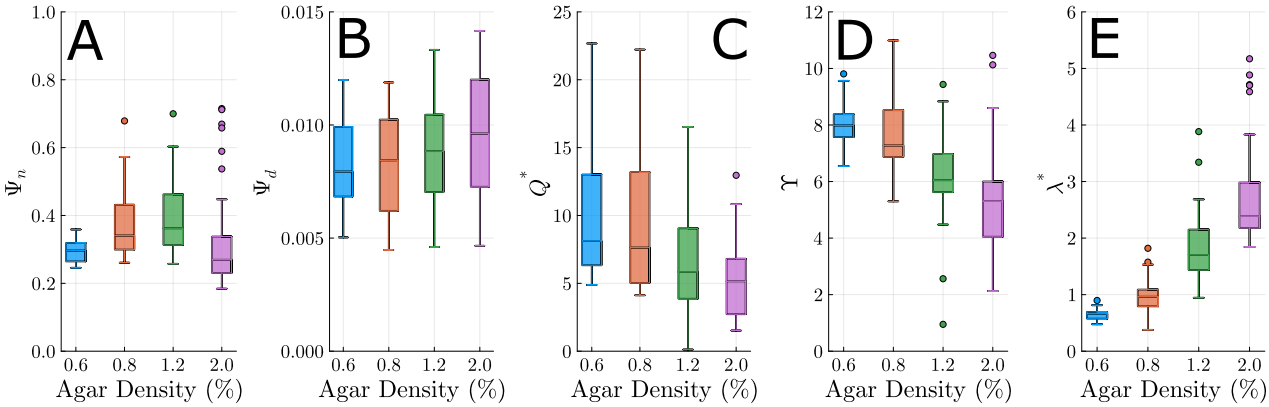

Figure 4.3: Box plots representing the optimal parameter values found using our parameter estimation procedure for 50 synthetic experiments on each agar density. Circle markers indicate outliers. (A) Ψ_n. (B) Ψ_d. (C) Q^*. (D) Υ. (E) λ^*.

For a fixed agar density, the optimal biomass production rates Ψ_n and cell death rates Ψ_d vary. This result demonstrates the variability across synthetic replicates. However, as expected both parameters do not depend strongly on agar density. Some studies have predicted increased cell death with increasing adhesion strength [89, 90]. Although we predict slightly higher values of Ψ_d on harder agar, we cannot reliably conclude that agar density affects Ψ_d in yeast from our available data. Our parameter estimates in Table 4.1 indicate that the nutrient uptake rate Q^* and nutrient consumption rate Υ both decrease with increasing agar density. Although agar hardness is thought to inhibit nutrient uptake in bacteria such as *E. coli* [56], we find wide variability in Q^* across the synthetic experiments. This finding is consistent with the parameter pair heat maps in Figure 4.2, where Q^* was a difficult parameter to identify. The decreasing trend in Υ with agar density is more robust than the trend in Q^*, but is still subject to significant variability. Of all parameters, the biofilm–substratum adhesion strength λ^* has the strongest variation with agar density in synthetic experiments, and the least variability across synthetic replicates. These results provide further evidence that agar density mediates friction between the colony biofilm and substratum in *S. cerevisiae* colony biofilms.

A characteristic of extensional flows is that the expansion speed depends on the initial condition [31, 36]. Since the functional form of $\mathcal{H}(x)$ and the initial thickness H_0 are unknown, we also investigate the effect of the initial thickness, H_0, on the results. Varying the initial thickness between zero and three *S. cerevisiae* cells thick reveals that the distance $\rho(\theta, a)$ is relatively insensitive to H_0, compared to other parameters. This is especially true for $H_0 \geq 0.05$, which is the dimensionless thickness of a single yeast cell. Therefore, the differences between replicates are unlikely to be due to experimental

variations in H_0. These results are presented in Figure 4.4.

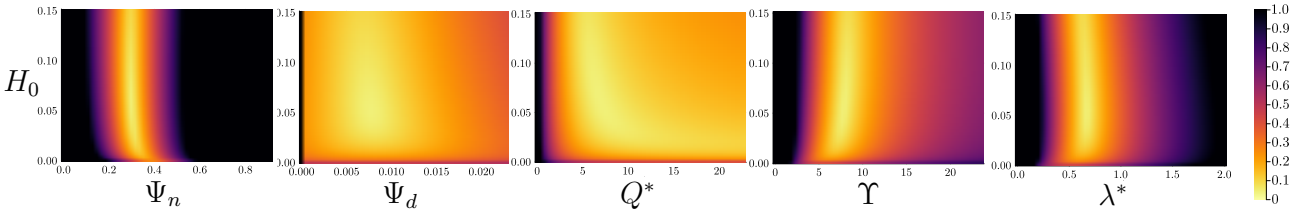

Figure 4.4: Parameter pair heat maps for the initial thickness, H_0, on 0.6% agar. Plots represent the distance between the numerical solution and experimental data, $\rho(\theta, a)$, for given parameter combination. Unless otherwise stated, parameters take the optimal values. When varied, each parameter ranges from zero to three times the optimal value (2,500 total simulations).

5 Conclusion

In this manuscript, we have combined *S. cerevisiae* experiments and mathematical modelling to quantify how agar density and cell death impact yeast colony biofilm growth. Our experimental method extended previous mat formation experiments [7–9] to incorporate rectangular geometry, colony biofilm composition measurements, and aspect ratio measurements. We adapted the thin-film extensional flow model of Tam et al. [36] to include cell death and resistance to slip caused by biofilm–substratum adhesion. Using new experimental data from multiple experiments, we estimated model parameters for four agar densities. The strongest impact of increasing agar hardness was to increase the biofilm–substratum adhesion strength, measured through the parameter λ^*. This finding is consistent with previous studies in bacteria, suggesting that increased adhesion with agar hardness is a general phenomenon of microbial growth. There was also a decrease in nutrient uptake and consumption rates on harder agar. These effects have also been hypothesised or observed in bacteria. However, parameter sensitivity analysis and synthetic data yielded wider variability in the nutrient uptake rate and consumption parameters compared to biofilm–substratum adhesion. The biomass production and death rates were consistent across agar densities.

Our mathematical model is based on the principle that the colony biofilm constituents can be modelled as viscous fluids [91]. Provided that this assumption is valid, the model describes the mechanics of colony biofilm growth due to biomass proliferation, mediated by a depleting nutrient supply. Although the slip parameter λ^* quantifies the drag due to biofilm–substratum adhesion, it does not provide a full description of the substratum mechanics. The agar may dehydrate over time [56], and colony biofilm attachment to the substratum may exert forces that deform the agar [54]. Our cross-sectional photographs of the colony biofilm indicate that these deformations might be small, but we neglect such deformations in the model. To confirm that agar deformation does not alter our main conclusions, a possible extension to the model could be to incorporate the agar mechanics explicitly. This would complicate the analysis significantly, as one would need to incorporate constitutive relations for the material properties of the agar, and continuity of stress on the biofilm–substratum interface, which may also change shape over time. This extension is left for future work.

A further extension for future work is to incorporate osmotic swelling, which influences bacterial biofilm expansion [32–34]. Despite evidence that osmotic stresses indeed affect individual *S. cerevisiae* cells [92],

the phenomenon of osmotic swelling in yeast colony biofilms is not widely reported. Osmotic influx from the substratum would contribute to volumetric growth of the colony biofilm, and nutrient supply. If significant, it would introduce new parameters to the model and alter the estimates of existing parameters. Another avenue for future investigation is to understand the non-uniform front pattern that often develops at later times, as shown in Figure 2.1. The amplitude of this pattern decreases on harder agar, and future work could focus on understanding how nutrient availability and agar density affect these interfacial instabilities.

Fungal biofilms are notorious for their ability to colonise indwelling medical devices [93, 94], and are a leading cause of hospital-acquired infections [95–98]. Our cross-sectional images reveal that a small number of yeast cells invade the agar medium in our experiments. Although the continuum model used in this paper does not permit invasion, invasive growth of yeast is important in clinical settings, because invasion of healthy tissue leads to the spread of infections [99]. Modelling the biophysics of yeast cell invasion is a problem for future work. Other colony biofilm experiments have featured a region of dead cells close to the leading edge. This region is thought to appear due to cells undergoing regulated cell death [22]. We neglected regulated cell death in this work, because our colony biofilms instead have regions of cell death near their inoculation site, reminiscent of a necrotic core [100, 101] formed by accidental cell death [22]. Since regulated cell death is known to occur in yeast colony biofilms, fully characterising the roles of accidental and regulated cell death [51], and their relationship to mechanics, remains an open question. In addition to multiple types of cell death, future work could also involve applying the multi-phase model to multi-species colony biofilms, which more closely resemble the biofilms found in healthcare environments. Natural and artificial surfactants can disrupt biofilm growth in wounds and hasten their removal [102]. Whilst this effect is irrelevant to sliding motility where surface tension is negligible, it provides another promising avenue for future modelling work, as researchers continue to explore the wide range of yeast growth modes and strategies for eliminating biofilms.

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Authorship Contribution Statement

- **AKYT:** Conceptualisation, methodology, software, validation, formal analysis, investigation, data curation, writing – original draft, writing – review & editing, visualisation, funding acquisition.
- **DJN:** Conceptualisation, formal analysis, writing – review & editing.
- **JMG:** Methodology, investigation, data curation, writing – original draft.
- **JZ:** Methodology, investigation, data curation.
- **CWG:** Conceptualisation, funding acquisition.
- **VJ:** Resources, writing – review & editing, supervision, funding acquisition.

- **BJB:** Conceptualisation, writing – review & editing, project administration, funding acquisition.
- **JEFG:** Conceptualisation, writing – review & editing, supervision, funding acquisition.

Competing Interests

We have no competing interests to declare.

Data Availability

Open source JULIA code and experimental photographs used to generate the results in this paper is available on GitHub.

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