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The relationship between cell density and cell count differs among Saccharomyces yeast species

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Abstract

There is a recent push to develop wild and non-domesticated *Saccharomyces* yeast strains into useful model systems for research in ecology and evolution, and for industrial and medical applications. Yet, the variation between species and strains in important population parameters, such as growth rate and carrying capacity, remains largely undescribed. Here, we investigated the relationship between two commonly used measures in microbiology to estimate growth rate – cell density and cell count - in 23 strains across all eight known *Saccharomyces* species with different ecological and geographic origins . We found that the slope of this relationship significantly differs among species. Thus, a given optical density (OD) does not translate into the same number of cells across species. We then speculated that this is due to species with smaller cells producing steeper slopes. While average cell size indeed differed between species, surprisingly, we found a slightly positive relationship between cell size and the slope of the cell density-cell count relationship, in the opposite direction than we predicted. Our results show that the strain- and species-specificity of the cell density and cell count relationship should be taken into account when running competition experiments requiring equal starting population sizes, when estimating the fitness of strains with different genetic backgrounds in experimental evolution studies, or when optimizing strains for industry. If we want to improve the biological interpretations of fitness data from wild yeasts, and draw meaningful conclusions from comparisons between wild and established laboratory strains, we need to calibrate our fitness estimates carefully.

Introduction

The genus *Saccharomyces* (Saccharomycetales, Ascomycota) originated ˜100 million years ago facilitated by whole genome duplication and an increased metabolic capacity to degrade sugar to ethanol . It currently comprises eight species, which diverged 4-20M years ago . All species are monophyletic with high levels of sequence collinearity but show vast genetic and ecological diversity . Recently, the genomic and ecological data available for all eight species (and their hybrids) have considerably grown , promoting yeast from a traditional laboratory model system that only included a few clonal strains to one in which we can answer questions relevant for ecology and evolution . For instance, we can now resolve the genetic basis of adaptation to environmental challenges , we can investigate which ecological factors determine the distribution and structure of yeast populations in the wild , and measure the level of divergence and gene flow between them . Our growing knowledge of the phenotypic and genetic diversity of wild yeasts also allows us to study important ecological traits comparatively, across species backgrounds, alleviating limitations resulting from only including a handful of well-characterized laboratory strains in our analyses. One ecological trait that stands out as particularly diverse between species are their temperature preference profiles . Resistance and adaptation to high or fluctuating temperatures are an increasing research focus for climate change biology. Temperature profiles are also important for the development of industrial strains, e.g. for fermentation products and biofuels . Of the eight species included here, five are considered cold-tolerant (*S. kudriavzevii, S. arboricola, S. uvarum* , *S. eubayanus* , and the recently discovered *S. jurei)* , one species is thermo-tolerant (*S. cerevisiae*), and two species are considered a thermo-generalist (*S. paradoxus* and*S. mikatae*) growing

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well in a broad range of temperatures .

Developing wild strains into effective systems for research and industry requires the systematic testing and measuring of fundamental population phenotypes including their growth rates, kinetics (e.g. the length of the lag phase), and yield. Microbial research often applies high-throughput methods to estimate population growth and fitness in environments of interest, e.g. media containing different nutritional and stress conditions. A common technique is to measure the optical density (OD) of microbial cultures using a spectrophotometer ('plate reader'). Optical density measures the turbidity of liquid cultures, which is assumed to be proportional to the cell number, i.e. the concentration of cells in the sample . Specifically, OD is the negative log of transmittance, i.e. the fraction of light detected when passed through a cuvette or micro-titer plate containing the microbial culture. It is typically measured at a wavelength of 600nm as this electromagnetic radiation is thought to not cause cell damage. Calculations follow the Beer-Lambert law , which states that OD is proportional to the concentration of a solution. However, this law only applies to cultures with low cell densities (typically OD_{600} up to 0.1). At higher densities, the light gets increasingly scattered between cells, and OD does not increase as fast as the cell titer. Using spectrophotometry to infer population fitness has additional limitations affecting the translation of OD into cell counts. Importantly, the method does not differentiate between dead and alive cells and the absorption coefficient (ε) can be affected by cell size (Fukuda 2023). Different methods exist for the calibration of OD measurements , including the use of silica microspheres, direct cell counting with microscopy, and colony counting in serial dilutions on agar plates . But the most efficient, high-throughput method is flow cytometry, using laser-based detection of individual cells that allows for accurate cell count estimates

Here, we explore the variation in the relationship between OD measures from spectrophotometry and cell counts from flow cytometry, across all eight *Saccharomyces* yeast species. To also test for variation within species, we used three strains per species (except for *S. jurei* , where only two strains were available) from different geographic and ecological origins, including isolates from fruit, soil, rotten wood, and tree bark from Europe, Asia, North and South America, and Australia (Table S1). We hope our study expands the knowledge base of important growth parameters of non-*cerevisiae* strains and improves the biological interpretation of population fitness data from wild, non-domesticated yeasts.

Materials and methods

Selection of strains

We selected a total of 23 strains (detailed strain information in Table S1). Each of the eight *Saccharomyces* species is represented by three strains isolated from different geographic locations and habitats, with the exception of the newly discovered *S. jurei* , for which only two strains were available to us. We only used non-domesticated, wild-type strains that do not show flocculation or cell clumping (Table S1).

Cell density and cell count measurements

We grew yeast strains from frozen glycerol stocks overnight in 5 mL liquid YNB complete medium (0.67% Yeast Nitrogen Base; 2% glucose). OD_{600} was measured with a spectrophotometer (BioTek Epoch 2) in 96-well plates with 200 µL yeast culture per well, in four dilutions with miliQ water (2, 2.5, 3.33, 5, 10 and 20-fold dilution) to provide a range of OD readings. Raw OD measurements were blank-corrected. The average OD of each strain was calculated from six technical replicates per dilution, with three independent read-outs obtained from the plate reader. To maintain linearity between cell number and OD_{600} values, OD must be within the dynamic range of equipment. However, OD frequently reaches values near 10 after 24 hours of cultivation at 25°C. In the current study, we therefore used an OD_{600} of 0.4 as the upper limit.

For flow cytometry, we used the same cultures that we had prepared for the OD_{600} measurements, and diluted them further in phosphate-buffered saline (PBS) with a 50X dilution factor in 96 well plates. The plate layout, i.e. the position of strains and replicates on the 96-well plates, were identical in both the spectrophotometer and flow cytometry runs.

Linear regressions between cell density (OD from spectrophotometry) and cell count (from flow cytometry)

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were performed. Goodness of fit (R^2) and 95% confidence intervals were calculated and plotted along the slope in GraphPad Prism 10. Species and strain-specific slopes were extracted as the change of Y per one unit increase in X (i.e. the cell count increasing per OD_{600}unit). We tested for differences between species in slopes, using ANOVA with Tukey HSD tests for pairwise comparisons in JMP (v17.2.0). To assess how much of the variance in slope is explained by differences between strains, we applied a mixed model using 'species' as fixed and 'strain' as random effect.

Cell size measurements

Strains were grown from frozen glycerol stocks overnight in 5 mL liquid YNB complete medium, and diluted using a 50X dilution factor. Cell photos were taken with a compound microscope (Leica^{TM}) at 100X magnification connected to a DSRL camera. Between 69 and 364 cells were measured per species. Photos were processed using the StarDist plugin for ImageJ/Fiji software, a cell detection method for microscopy images, and processed using ImageJ/Fiji's 'find edges' option. We used the detection model DSB 2018

settings at 80 for probability/score threshold and 70 for overlap threshold. Cell area was measured and log transformed and a mixed model using 'species' as fixed effect and 'strain' as random effect was used on cell size as a response variable in JMP (v17.2.0).

Results

The relationship between optical density and cell number differs between species

To investigate the relationship between cell count (obtained from flow cytometry) and OD_{600} -value (from spectrophotometry) across all 23 strains, we used linear regression (Figure 1, all $R^2 > 0.87$, $p < 0.01$). We found significant differences between species in the slope of this relationship (ANOVA: $F_{7,22} = 5.63$, p = 0.0025; Figure 2). The average slope extracted from *S. cerevisiae* regressions was significantly steeper than the slopes found in *S. mikatae,S. jurei, S. arboricola* , and *S. uvarum* (Tukey HSD, all$p < 0.05$). The random effect 'strain' was non-significant and only explained 4% of the variation in the data (Wald $p = 0.81$; Table S2).

Yeast species significantly differ in cell size

We observed significant differences in average cell size (log cell area in μm^2) between species (mixed model output for fixed effect 'species': $F_7 = 14.17$, $p < 0.0001$; Figure 3). Especially *S. arboricola* , *S. eubayanus,* and*S. uvarum* cells are significantly smaller than the cells of all other species, while *S. cerevisiae* and *S. paradoxus* have significantly larger cells than most other species (Figure 3). The random effect 'strain' explained a small (7.1%) but significant proportion of the variance in cell size (Wald $p = 0.041$, Table S3). Indeed, most species showed some significant differences in cell size between strains (ANOVA for all species/t-test for *S. jurei* : all $F/t > 3.40$, all $p < 0.04$), except for the three *S. arboricola* strains, which were more uniform in size.

Cell size explains variation in the cell density - cell count relationship

We expected cell size to inversely predict the steepness of slopes extracted from cell density - cell count regressions, because a given optical density may result in fewer cell counts if cells are on average larger. However, we found the opposite: the steepness of strain-specific slopes extracted from cell density - cell count regressions increased slightly with increasing strain cell size ($R^2 = 0.24$;$p = 0.0223$; Figure 4).

Discussion

We assessed the linearity between two common measurements for the size of yeast populations in laboratory cultures, cell density (OD, obtained from spectrophotometry) and cell count (obtained from flow cytometry), across all eight species of *Saccharomyces* (Figure 1). The steepness of the slope of this relationship is informative for researchers conducting studies across species and strain backgrounds, comparing the competitive fitness of stains, or specific aspects of their growth kinetics. We found that the average slope of the relationship between cell density and cell count significantly differs between species. Especially *S. cerevisiae* has a

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significantly steeper slope than most other species, i.e. they produce higher cell counts at lower OD values. Interestingly, the patterns in slope variation that we observed across the genus, closely follow the phylogenetic relationship of the species, with more closely related pairs (*S. cerevisiae* /*S paradoxus* , *S. mikatae* /*S. jurei* , and *S. eubayanus* /*S. uvarum*) having more similar slopes (Figure 2). This suggests that the species- and strain- specificity of this relationship should be taken into account when setting up and interpreting results of competitive fitness assays, or when fine-tuning cell titers and inocula of non-commercial strains for industrial applications. It also shows the limitations of using only a single laboratory *S. cerevisiae* strain as a reference point when exploring the population biology of wild strains.

We then speculated that differences in cell size may explain variation in the steepness of the slopes between species. Variation in cell size is expected, given the large diversity of ecological niches these species inhabit (Table S1), and has been described in *S. cerevisiae* as a result of temperature and nutrient availability (Kellogg & Levin, 2022), affecting basic physiological functions such as protein synthesis and cell division rate . The intensity and the radius of light scattering in the spectrophotometer depends on cell size, thus affecting the absorbance of the microbial culture . Indeed, we found that species significantly differ in average cell size, with*S. eubayanus* having the smallest (1.16 ± 0.12 log μm^2) and *S. cerevisiae* having the largest cells (1.4 ± 0.11 log μm^2, Figure 3). But against our expectation, that strains with larger average cells would give lower cell counts at similar OD readings, increasing cell size did not predict a decrease in slopes from cell count – cell density regressions. Instead, we found the opposite to be true. Slopes increased slightly with increasing cell size (Figure 4), suggesting that at a given OD-value, strains with larger cells also produce higher cell counts.

Besides cell size, other species-specific cellular features may affect the cell count – cell density relationship. The species investigated here are ecologically and genetically vastly divergent and likely differ in the composition of their cell wall, determining its strength and rigidity . For instance, chitin, chitosan, and cell wall proteins have been shown to have different refractive indices that can affect OD readings . Wall-resident proteins have diversified rapidly over evolutionary time in *Saccharomyces* as a result of gene silencing through epigenetic mechanisms and environmentally induced expression regulation, providing adaptability to different habitats and lifestyles . Chitin and chitosan are also known to vary in structure between fungal species . Besides cell wall composition, the number and structure of bud scars may also affect the cell's refractive index . Mother cells accumulate chitinous scar tissue from cytokinesis over their lifetime and different growth conditions (poor *vs* . rich media) can lead to variation in bud scar number . If species vary in the average number or structure of bud scars a mother cell carries (e.g. due to heritable differences in cell longevity or species-specific responses to nutrient availability), this may change their refractive indices and affect the translation of optical density readings into cell counts.

Conclusions

While spectrophotometry is widely used to assess the growth and fitness of microbial cultures, it has important limitations. Flow cytometry is a more accurate method for measuring population size but it is not as widely available or as cost-effective as spectrophotometry. Our work shows that the relationship between cell density (from spectrophotometry) and cell count data (from flow cytometry) differs among *Saccharomyces* yeast species. Thus, when conducting research on non-model strains and species of yeast and flow cytometry is not available, we caution to calibrate cell density measurements carefully (e.g. by manual cell or colony counting) prior to comparative fitness analysis, and to take in account the diverse ecology, life history, and physical properties of yeast species to avoid biological misinterpretations.

Acknowledgements

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Conflict of Interest Statement

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All authors declare that there is no conflict of interest related to this article.

References

Figure Legends

Figure 1 Linear regression of cell count (obtained from flow cytometry as number of cells/µL) on optical density (obtained from spectrophotometry as OD_{600}) across eight Saccharomyces species. Coloured lines represent three genetically different strains per species (except for S. jurei, where only 2 strains were available). Strain names are indicated on each species plot. Dotted lines are 95% confidence intervals.

Figure 2 *Slopes of eight Saccharomyces species extracted from regressions of cell counts (obtained from flow cytometry) on optical density (OD_{600}). Slopes are averaged across strains within species and expressed as the cell count increasing per OD_{600} unit. Bars represents 95% confidence intervals from regression of all data per species. Levels not connected by same letter are significantly different. The species' phylogenetic relationship is shown at the bottom.*

Φιγυρε 3 *Αεραγε ςελλ σιζε οφ 23 στραινς αςροσς ειγητ Σαςςηαρομψςες σπεςιες. Α) Ιμαγες οφ ψεαστ ςελλς ατ 100ξ μαγνιφισατιον. Β) ϊολιν πλοτ σηοωινγ τηε διστριβυτιον οφ ςελλ σιζε (λογ ςελλ αρεα ιν μm^2). Αεραγε ςελλ σιζε περ σπεςιες ις σηοων ατ τηε βοττομ. Τηε δαρκερ τηε ιολιν πλοτς, τηε σμαλλερ ςελλς αρε ον αεραγε. Λεελς νοτ ςοννεςτεδ βψ σαμε λεττερ αρε σιγνιφισαντλψ διφφερεντ ιν παιρωισε Τυκεψ ΗΣΔ τεστς αφτερ ΑΝΟΫΑ οφ 'σπεςιεσ΄ον 'λογ ςελλ αρεα΄ ($\Phi_{7,1710}$= 96.12, π < 0.001).*

Figure 4 Scatter plot of strain-specific slopes from linear regressions of cell count (obtained from flow cytometry as number of cells per µL) on optical density (obtained from spectrophotometry as OD_{600}) against average strain cell size (log cell area in μm^2).

Figures

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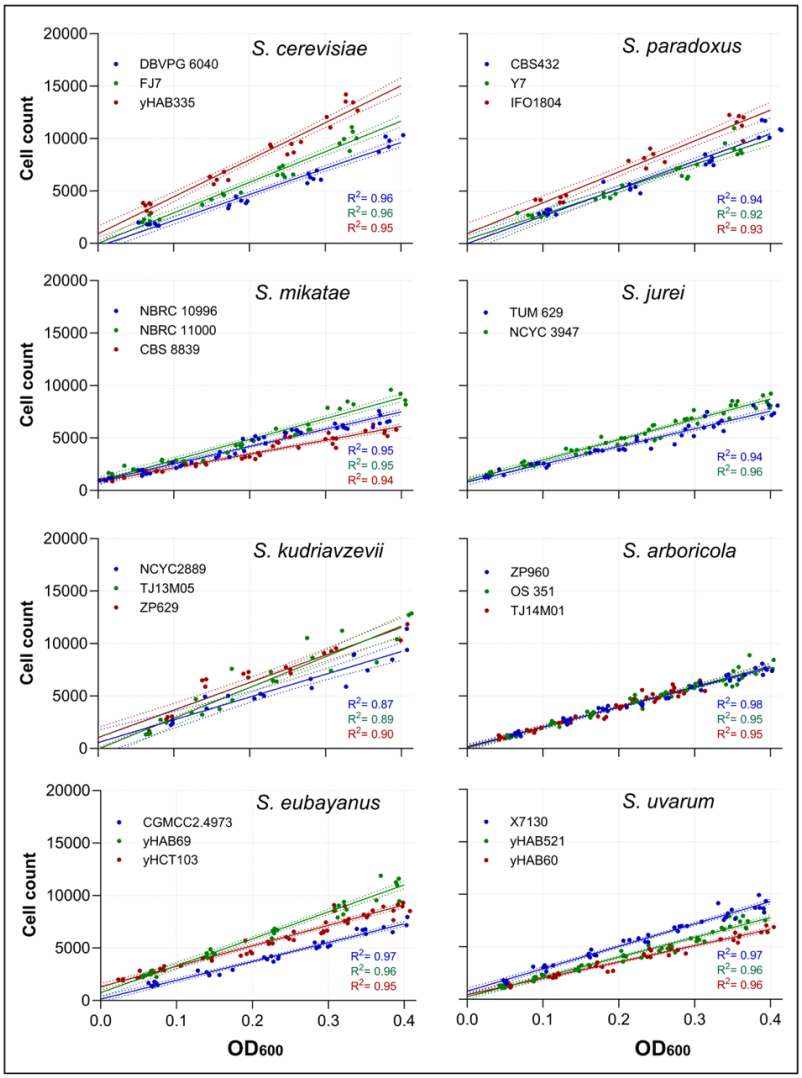

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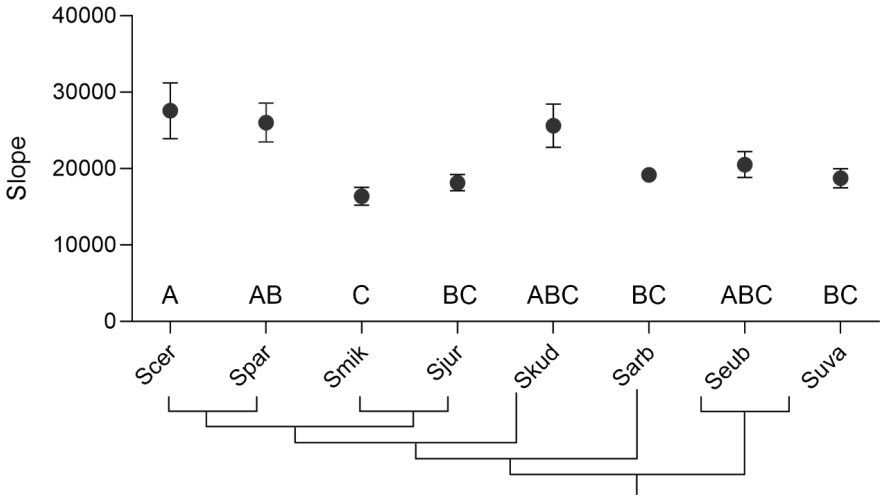

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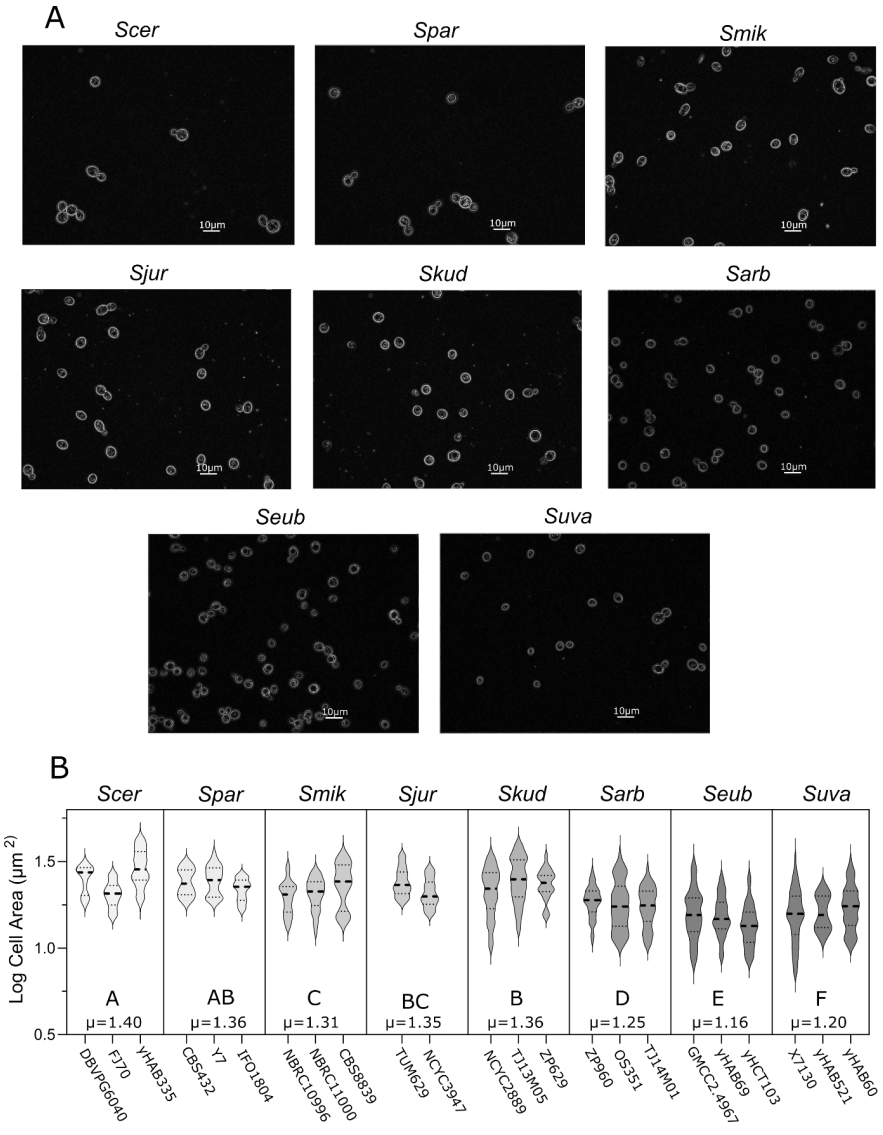

Φιγυρε 3 *Αεραγε σελλ σιζε οφ 23 στραινς ασροσς εινητ Σαχχηαρομψχες σπεσιες. Α) Ιμαγες οφ ψεαστ σελλς ατ 100ξ μαγνιφισατιον. Β) ϝολιν πλοτ σηοωινγ τηε διστριβυτιον οφ σελλ σιζε (λογ σελλ αρεα ιν μμ^2). Αεραγε σελλ σιζε περ σπεσιες ις σηοων ατ τηε βοττομ. Τηε δαρκερ τηε ιολιν πλοτς, τηε σμαλλερ σελλς αρε ον αεραγε. Λεελς νοτ χοννεχτεδ βψ σαμε λεττερ αρε σγνιφισαντλψ διφφερεντ ιν παιρωισε Τυκεψ ΗΣΔ τεστς αφτερ ΑΝΟ″Α οφ 'σπεσιεσ′ ον 'λογ σελλ αρεα′ ($\Phi_{7,1710}$= 96.12, $\pi < 0.001$).*

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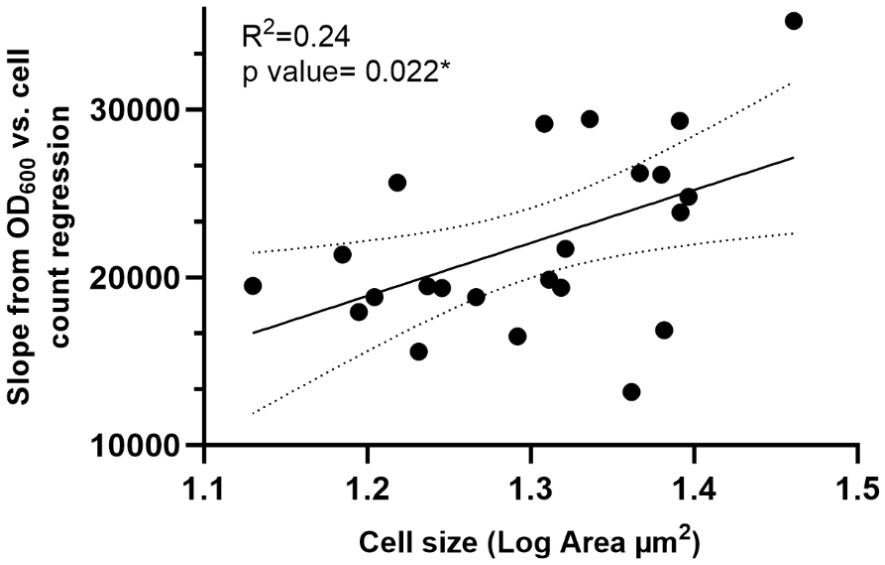

Figure 4 Scatter plot of slopes from linear regressions of cell count (obtained from flow cytometry as number of cells per µL) on optical density (obtained from spectrophotometry as OD_{600}) against average cell size (log cell area in μm^2) per strain.