Research Square

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Influence of Environmental and Nutritional Conditions on Nakaseomyces glabratus Biofilm Development In Vitro

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Abstract

Biofilm formation is a major virulence trait in *Nakaseomyces glabratus*, contributing to antifungal tolerance and persistent infections. Environmental and nutritional parameters influence fungal biofilm development, but standardized experimental frameworks for evaluating *N. glabratus* biofilm modulation remain limited. This study investigated how defined environmental conditions, including incubation time, agitation, and carbon source supplementation modulate biofilm development under controlled in vitro conditions. Biofilms were formed for 24 or 72 h under static (0 rpm) and dynamic (120 rpm) conditions in Sabouraud dextrose broth supplemented with glucose or galactose as carbon sources. Biofilm formation was assessed using complementary analytical methods, including crystal violet staining for total biomass, XTT reduction assay for metabolic activity, viable cell counts, and scanning electron microscopy for structural characterization. Incubation time and culture medium composition significantly affected biofilm development, whereas agitation exerted a comparatively minor effect. High carbon supplementation acted as a controlled metabolic challenge, enabling the investigation of biofilm adaptability under carbon-excess conditions. Overall, this study establishes a reproducible in vitro framework for investigating environmental and nutritional modulation of *N. glabratus* biofilms and provides a basis for future functional and antifungal studies.

Introduction

Biofilms are structured microbial communities embedded in a self-produced extracellular matrix and attached to biotic or abiotic surfaces [1]. This mode of growth provides enhanced protection against environmental stress, host immune responses, and antimicrobial agents, playing a central role in the persistence and recurrence of infectious diseases [2]. In clinical settings, biofilm-associated microorganisms are frequently implicated in device-related infections, mucosal colonization, and chronic inflammatory conditions, representing a major challenge for effective antimicrobial therapy [3].

Nakaseomyces glabratus (formerly *Candida glabrata*) is an opportunistic yeast increasingly associated with healthcare-related infections, particularly in immunocompromised and hospitalized patients [4, 5]. Unlike *Candida albicans*, *N. glabratus* does not form true hyphae but exhibits remarkable tolerance to environmental stressors and antifungal agents, partly mediated by its ability to form biofilms [6–8]. These biofilms contribute to therapeutic failure and recurrent infections, emphasizing the need to better understand the environmental and nutritional factors that modulate their development [9].

Biofilm formation in *N. glabratus* is a dynamic and highly regulated process influenced by multiple physicochemical and nutritional parameters, including pH, oxygen availability, shear forces, and carbon source composition [10–12]. Carbon metabolism, in particular, plays a pivotal role in fungal adhesion, extracellular matrix production, and stress adaptation [13, 14]. Previous studies have demonstrated that different carbohydrates may differentially affect growth, virulence, and antifungal susceptibility in *Candida* species [10, 13, 15]. However, data addressing how defined culture conditions modulate *N. glabratus* biofilm development remain limited.

In vitro biofilm models commonly employ nutrient-rich media and controlled environmental challenges to ensure reproducible biomass formation and sufficient signal-to-noise for quantitative and microscopic analyses [16–18]. Within this framework, high carbon supplementation may serve as a proof-of-principle metabolic challenge to enhance phenotypic contrast and to probe biofilm adaptability under carbon-excess conditions, rather than to reproduce host physiological environments. Such standardized approaches are particularly useful for comparative studies and for developing reliable models to test antifungal agents and environmental influences on biofilm formation.

Therefore, the present study aimed to investigate how defined environmental and nutritional parameters —including incubation time, agitation speed, and glucose or galactose supplementation—modulate *N. glabratus* biofilm development under controlled in vitro conditions. Biofilms were characterized in terms of biomass, metabolic activity, viable cell counts, and ultrastructural organization in order to establish a reproducible framework for environmental modulation studies. The null hypothesis was that these experimental parameters would not significantly influence biofilm development.

Materials and Methods

Strain and culture media

The reference strain *Nakaseomyces glabratus* ATCC 90030 was used throughout the study. Stock cultures were maintained in Sabouraud dextrose broth (SDB; Difco, Le Pont de Claix, France) supplemented with 20% (v/v) glycerol at − 80°C. Prior to each experiment, the strain was subcultured on Sabouraud dextrose agar (SDA; Difco) and incubated at 37°C for 24 h.

Colonies were transferred to SDB and incubated at 37°C for 24 h under orbital agitation (120 rpm). Cells were harvested by centrifugation (8000 × g, 5 min), washed twice with phosphate-buffered saline (PBS; 0.1 mol/L, pH 7.0), and resuspended in fresh SDB. Cell density was adjusted to 1×10^7 cells/mL using a Neubauer hemocytometer.

Three culture media were evaluated: SDB alone, SDB supplemented with 500 mmol/L glucose (GLU; Sigma-Aldrich, St. Louis, MO, USA), or SDB supplemented with 500 mmol/L galactose (GAL; Sigma-Aldrich). This concentration was selected to impose a controlled carbon-excess condition and to enhance phenotypic discrimination across complementary assays, rather than to mimic physiological carbohydrate levels.

Biofilm formation

Biofilms were formed in sterile, flat-bottom 96-well polystyrene microplates (Costar, Corning Inc., Tewksbury, MA, USA). Aliquots of 200 µL of standardized cell suspension were added to each well. Plates were incubated at 37°C for 24 or 72 h under static (0 rpm) or dynamic (120 rpm) conditions in an

orbital shaker. The culture medium was gently replaced every 24 h to ensure nutrient availability. After each incubation period, wells were washed once with PBS to remove non-adherent cells.

Total biomass quantification (crystal violet assay)

Biofilm biomass was quantified using the crystal violet (CV) staining method. After washing, biofilms were fixed with 200 µL of 99% methanol (Sigma-Aldrich) for 15 min and air-dried. Subsequently, 200 µL of 0.1% (w/v) crystal violet solution was added to each well and incubated for 5 min at room temperature. Excess dye was removed by gentle rinsing with distilled water. After drying, bound crystal violet was solubilized with 200 µL of 33% (v/v) acetic acid. The resulting solution was transferred to a new microplate, and absorbance was measured at 540 nm using a microplate spectrophotometer (SpectraMax i3x, Molecular Devices, San Jose, CA, USA) [19].

Metabolic activity quantification (XTT assay)

Metabolic activity was assessed using the XTT reduction assay. A freshly prepared solution containing XTT (0.5 mg/mL) and menadione (1 µmol/L) (Sigma-Aldrich) was used. After washing, 200 µL of XTT-menadione solution was added to each well, followed by incubation at 37°C for 3 h in the dark under gentle agitation (120 rpm). Subsequently, 100 µL of the supernatant was transferred to a new microplate, and absorbance was measured at 490 nm. Background absorbance from cell-free wells was subtracted prior to analysis [19].

Given the known low tetrazolium-reducing capacity of *N. glabratus*, XTT results were interpreted as complementary indicators of metabolic activity and not as standalone viability measures.

Counting of Colony-forming units (CFU)

For viable cell enumeration, biofilms were mechanically disrupted by scraping and vigorous pipetting. The resulting suspensions were serially diluted in PBS, and aliquots were plated on SDA. Plates were incubated at 37°C for 24–48 h, and colonies were counted to determine colony-forming units (CFU). Results were expressed as $\log_{10}$ CFU/cm² [19].

Scanning electron microscopy (SEM) analysis

For morphological analysis, biofilms were formed in 24-well plates under static conditions for 24 or 72 h. After washing with PBS, samples were fixed with 2.5% (v/v) glutaraldehyde in 0.1 mol/L cacodylate buffer for 2 h at room temperature. Specimens were rinsed and dehydrated through a graded ethanol series (30%, 50%, 70%, 90%, and 100%), followed by critical-point drying. Samples were mounted on aluminum stubs, sputter-coated with gold, and examined using a field-emission scanning electron

microscope (FEG-VP Supra 35; Carl Zeiss, Jena, Germany). Representative micrographs were obtained for qualitative analysis.

Statistical analysis

All experiments were performed in triplicate in three independent assays. Data were normalized by well surface area. Normality and homoscedasticity were assessed using the Shapiro–Wilk and Levene's tests, respectively. A three-way analysis of variance (ANOVA) was performed to evaluate the effects of incubation time, agitation speed, and culture medium, followed by Tukey's post hoc test ($\alpha = 0.05$). Statistical analyses were conducted using R software (R Foundation for Statistical Computing, Vienna, Austria).

Results

Effects of incubation time, agitation, and culture medium on biofilm biomass

Total biomass measurements revealed a significant three-way interaction among incubation time, agitation speed, and culture medium ($p < 0.05$) (Fig. 1). At 24 h under static conditions, biofilms grown in GLU medium exhibited significantly lower biomass than those formed in GAL medium. Under dynamic conditions, SDB alone resulted in the lowest biomass at this time point.

At 72 h under static conditions, biofilms formed in SDB and GLU medium showed significantly higher biomass than those grown in GAL medium. Overall, agitation did not markedly affect biomass accumulation, except for 24-h biofilms grown in GLU medium, which exhibited increased biomass under dynamic conditions.

Effects of experimental conditions on metabolic activity

Metabolic activity, assessed by the XTT assay, was significantly influenced by incubation time and culture medium ($p < 0.05$) (Fig. 2). GAL consistently promoted higher XTT reduction compared with the other media, particularly at 72 h. No statistically significant differences were observed between static and dynamic conditions.

Despite the low tetrazolium-reducing capacity of *N. glabratus*, reproducible trends were observed across independent experiments, supporting the comparative interpretation of metabolic activity under the tested conditions.

Influence of growth conditions on viable cell counts

Quantification of viable cells demonstrated that incubation time was a major determinant of biofilm development ($p < 0.05$) (Fig. 3). In general, 72-h biofilms exhibited significantly higher CFU counts than 24-h biofilms, regardless of agitation speed.

GLU resulted in significantly lower CFU values compared with SDB alone at most time points, whereas GAL did not markedly suppress cultivable cell numbers. Agitation exerted no consistent effect on CFU counts across the evaluated conditions.

Ultrastructural organization of biofilms

Scanning electron microscopy revealed time-dependent changes in biofilm architecture (Fig. 4). At 24 h, biofilms consisted mainly of loosely organized clusters of yeast cells with limited extracellular matrix. After 72 h, more compact cellular aggregates were observed, indicating progressive structural maturation.

No consistent morphological differences were detected among the tested culture media. Under all conditions, biofilms were composed predominantly of yeast-form cells partially embedded in an extracellular matrix.

Discussion

The present study investigated how defined environmental and nutritional conditions influence the development of *N. glabratus* biofilms under controlled in vitro conditions. The results indicate that incubation time and culture medium composition displayed the strongest influence on biofilm maturation, whereas agitation had only a limited effect under the experimental conditions employed. By applying a carbon-excess framework as a controlled metabolic challenge, this model allowed the assessment of biofilm adaptability under defined environmental conditions rather than attempting to reproduce host physiological conditions. These results highlight the importance of standardized experimental parameters for reproducible biofilm analyses and comparative studies.

Incubation time emerged as a key determinant of biofilm maturation. Although 72-h biofilms displayed higher viable cell counts, biomass values at 24 h were comparable or even higher under certain conditions. This apparent discrepancy suggests that extracellular matrix production and early adhesion events may reflect the rapid establishment of adhesion and extracellular matrix production during early stages of biofilm formation in *N. glabratus*, followed by progressive increases in viable cell density over time. Similar patterns have been reported for other *Candida* species, where early biofilm consolidation contributes to surface persistence and protection from environmental stress.[20–22].

The limited influence of agitation observed in this study is likely related to the low shear forces generated in microplate-based systems. While moderate fluid flow can enhance nutrient diffusion and oxygen availability, the experimental setup employed here may not have been sufficient to produce biologically relevant mechanical stress. Consequently, static and dynamic conditions yielded largely comparable biofilm phenotypes, consistent with previous in vitro studies using similar platforms [18, 23, 24].

Carbon source availability is widely recognized as a major determinant of fungal metabolism, stress adaptation, and virulence [24–26]. In the present study, glucose supplementation (GLU) was associated

with reduced viable cell counts, whereas GAL promoted higher metabolic activity at later stages of biofilm development. These observations suggest differential metabolic responses to distinct carbon sources, reflecting the metabolic flexibility of *N. glabratus*. Similar responses have been described in other fungal species, where carbon metabolism influences cellular fitness, stress tolerance, and biofilm behavior [27–29]. These metabolic differences are consistent with known regulatory pathways involved in fungal adaptation to carbon availability, including modulation of oxidative stress responses, mitochondrial activity, and carbon sensing mechanisms such as the cAMP–PKA signaling pathway [25–27, 29]. Although not directly assessed, these mechanisms may contribute to the observed differences in biofilm development under distinct nutritional conditions.

Importantly, the high sugar concentration employed in this study was not intended to reproduce physiological conditions but to impose a controlled carbon-excess challenge. This proof-of-principle approach aimed to enhance phenotypic contrast and improve the detection of biofilm responses across complementary assays. Nutrient-rich media are frequently employed in in vitro biofilm research to ensure reproducible biomass formation and to support downstream analyses such as antifungal susceptibility testing [16, 18]. Therefore, the present findings should be interpreted within the context of a controlled experimental framework rather than as a direct representation of host conditions.

Metabolic activity was assessed using the XTT assay. However, this method has recognized limitations when applied to *N. glabratus* due to its relatively low tetrazolium-reducing capacity. This phenomenon has been attributed to interspecies variability in tetrazolium metabolism among *Candida* species [30, 31]. For this reason, XTT data were interpreted as complementary indicators of metabolic activity rather than direct measures of viability. The combined assessment of biomass, CFU counts, and ultrastructural characteristics therefore provided a more comprehensive evaluation of biofilm development, and the consistency observed among these parameters supports the robustness of the experimental model [32, 33].

Scanning electron microscopy revealed relatively simple biofilm architectures characterized by clusters of yeast cells embedded in a sparse extracellular matrix. These findings are consistent with previous reports indicating that *N. glabratus* forms less complex biofilms than filamentous *Candida* species [34]. The moderate structural organization observed even after prolonged incubation may reflect intrinsic species-specific traits and reinforce the importance of integrating quantitative and structural data when evaluating biofilm maturation in this organism [16, 32].

This study has limitations that should be acknowledged. First, no functional assays, antifungal susceptibility tests, or gene expression analyses were performed, which limits the ability to establish direct mechanistic or clinical correlations. Second, the use of a single reference strain may not capture the full phenotypic variability of clinical isolates. Therefore, the findings should be interpreted within the context of a controlled in vitro model designed to assess environmental modulation rather than to infer virulence or antifungal tolerance mechanisms.

In conclusion, this study establishes a reproducible in vitro model for evaluating how environmental and nutritional parameters modulate *N. glabratus* biofilm development. Incubation time and carbon source composition were identified as key factors influencing biofilm maturation, while agitation had a limited effect under the conditions tested. The findings should be interpreted within the context of a controlled experimental framework and provide a basis for future studies incorporating functional and mechanistic analyses.

Declarations

Author contributions

GPS (Investigation, Visualization; Writing – original draft), PFCV (Visualization; Writing – original draft), DRM (Conceptualization, Supervision, Methodology, Validation, Formal analysis, Writing – review & editing), JPP (Resources, Validation, Writing – review & editing), ACBD (Resources, Funding acquisition), RLP (Formal analysis, Software), LSA (Conceptualization, Project administration, Data curation, Supervision, Writing – original draft; Writing – review & editing).

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Competing Interests

The authors have no competing interests to declare.

Ethical Approval

This study did not involve human participants, animals, or clinical samples. All experiments were conducted using commercially available microbial strains under standard laboratory conditions. Therefore, ethical approval was not required.

Data Availability Statement

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

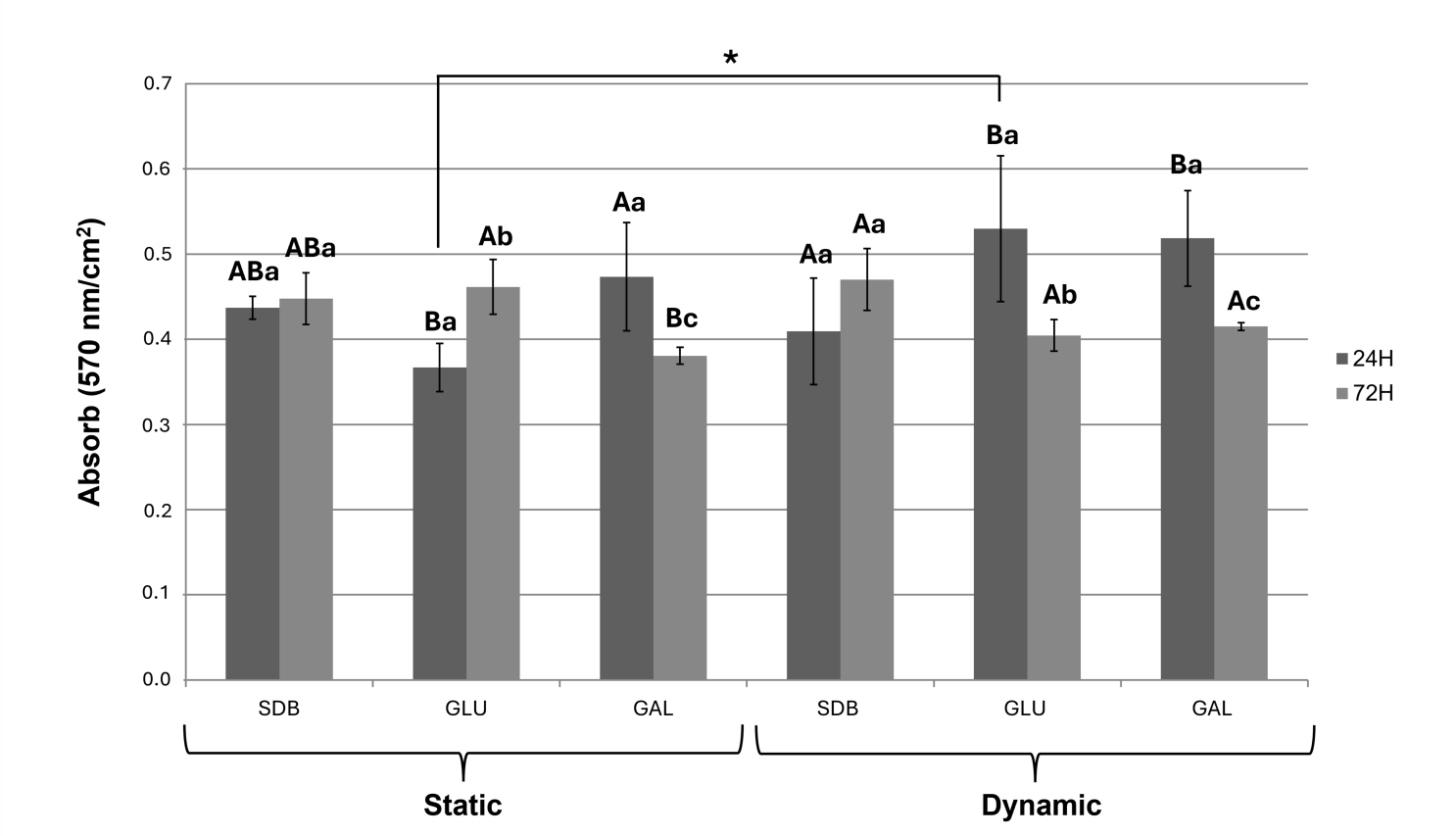


Figure 1

Mean absorbance per cm² for total biomass quantification of *Nakaseomyces glabratus* biofilms formed at 0 vs. 120 rpm during 24 or 72 hours in Sabouraud dextrose broth (SDB), SDB supplemented with either 500 mmol/L glucose (GLU) or 500 mmol/L galactose (GAL). Bars represent the standard deviations of the means. Different uppercase letters indicate significant differences among culture media for each biofilm formation period and agitation speed. Different lowercase letters indicate significant differences among biofilm formation periods within each culture medium and agitation speed. A significant difference between agitation speeds was observed for each culture medium and biofilm formation period (three-way ANOVA followed by Tukey's post hoc test, $p < 0.05$).

Alt text: Bar graph showing total biofilm biomass of *Nakaseomyces glabratus* formed for 24 and 72 h under static (0 rpm) and dynamic (120 rpm) conditions in SDB, GLU, and GAL media. Bars represent mean absorbance values per cm² at 540 nm with standard deviation.

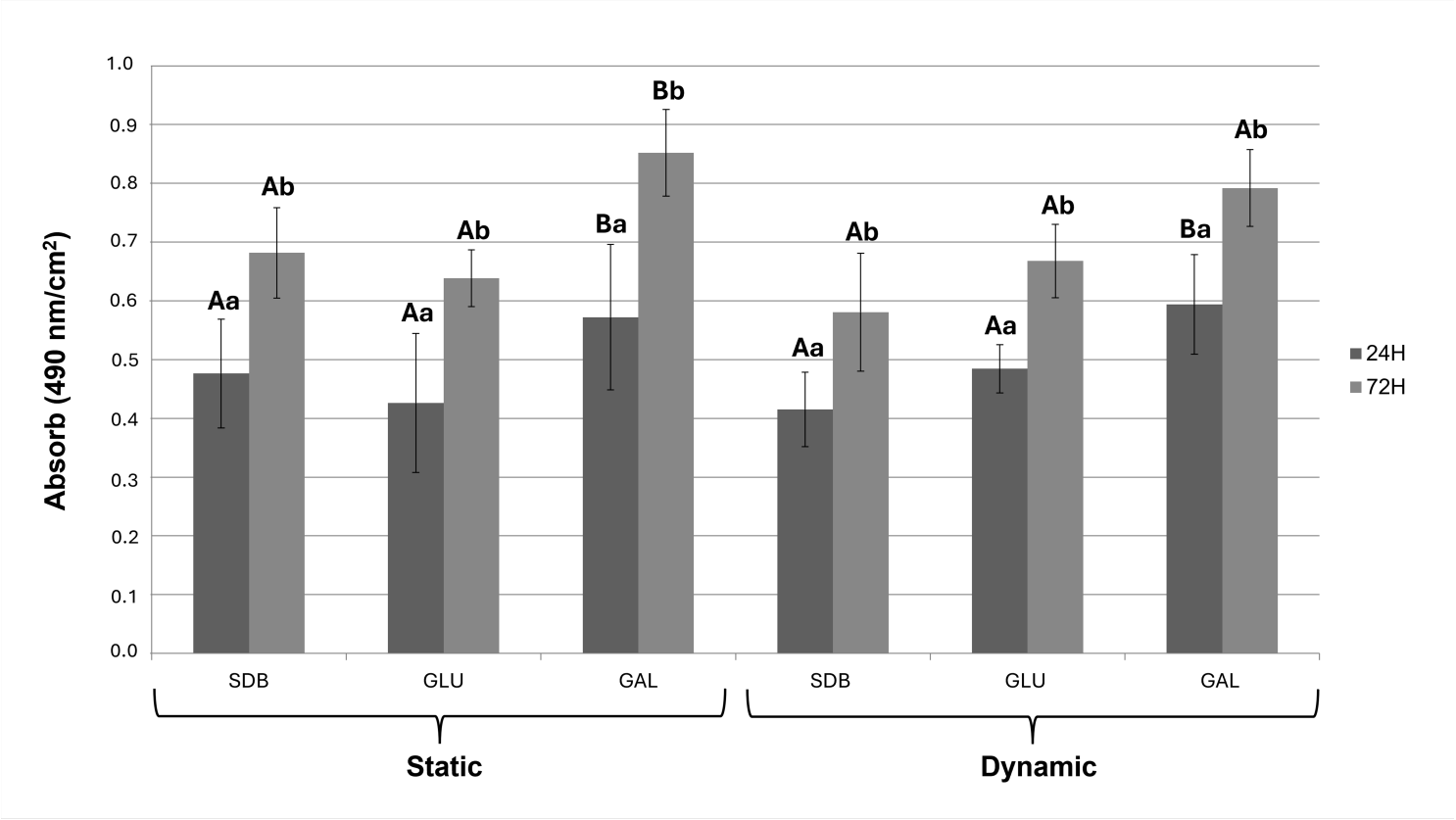


Figure 2

Mean absorbance per cm² for metabolic activity evaluation of *Nakaseomyces glabratus* ATCC 90030 biofilms formed at 0 vs. 120 rpm during 24 or 72 hours in Sabouraud dextrose broth (SDB), SDB supplemented with either 500 mmol/L glucose (GLU) or 500 mmol/L galactose (GAL). Bars represent the standard deviations of the means. Different uppercase letters indicate significant differences among culture media for each biofilm formation period. Different lowercase letters indicate significant differences among biofilm formation periods within each culture medium (three-way ANOVA followed by Tukey's post hoc test, $p < 0.05$).

Alt text: Bar graph displaying metabolic activity of *Nakaseomyces glabratus* ATCC 90030 biofilms measured by XTT reduction at 490 nm after 24 and 72 h under static and dynamic conditions in SDB, GLU, and GAL media, with error bars indicating standard deviation.

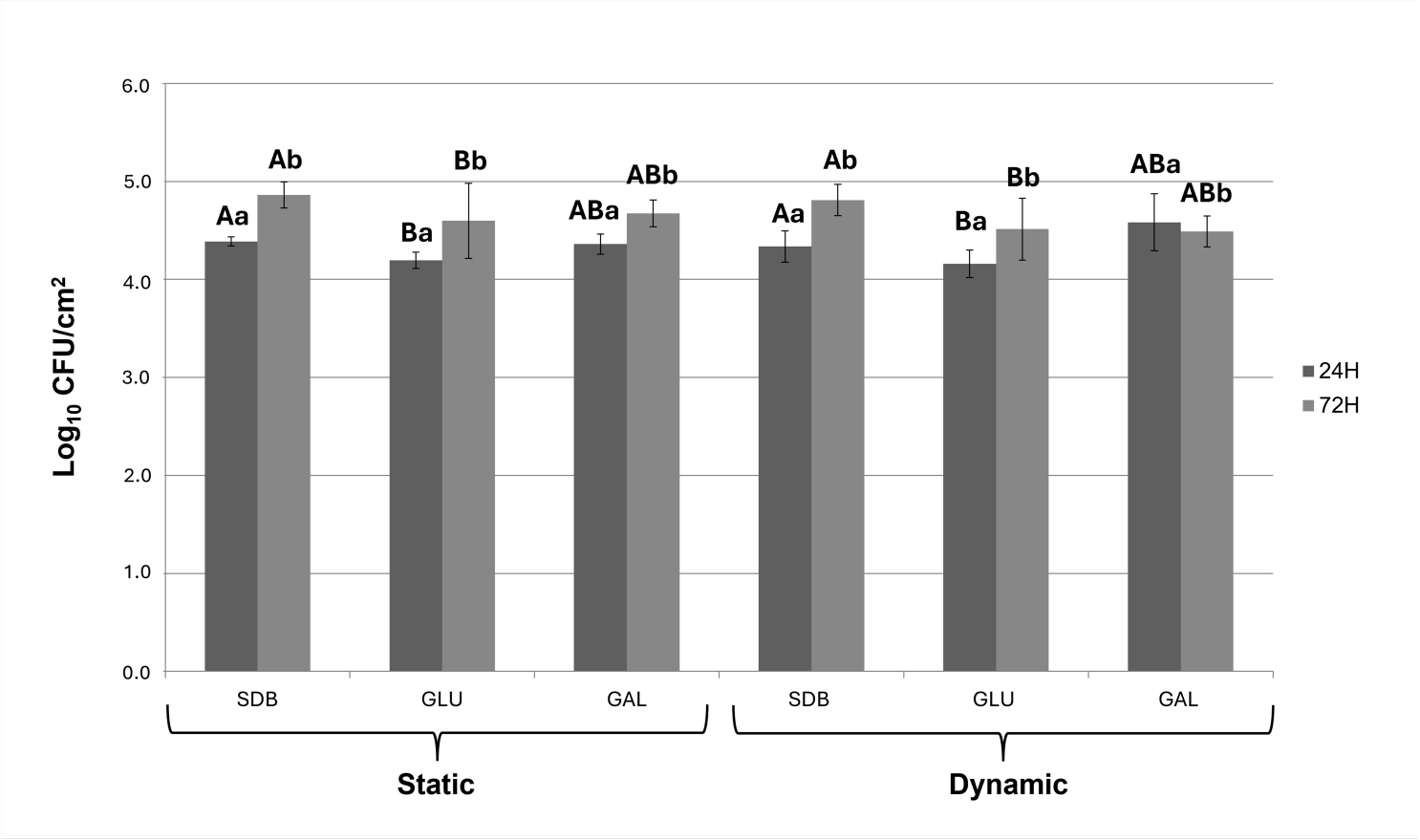


Figure 3

Mean logarithm of colony-forming units per cm² (log_{10} CFU/cm²) for *Nakaseomyces glabratus* biofilms formed at 0 vs. 120 rpm during 24 or 72 hours in Sabouraud dextrose broth (SDB), SDB supplemented with either 500 mmol/L glucose (GLU) or 500 mmol/L galactose (GAL). Bars represent the standard deviations of the means. Different uppercase letters indicate significant differences among culture media for each biofilm formation period. Different lowercase letters indicate significant differences among biofilm formation periods within each culture medium (three-way ANOVA followed by Tukey's post hoc test, $p < 0.05$).

Alt text: Bar graph showing viable cell counts of *Nakaseomyces glabratus* biofilms expressed as log_{10} CFU/cm² after 24 and 72 h under static and dynamic conditions in SDB, GLU, and GAL media. Error bars indicate standard deviation.

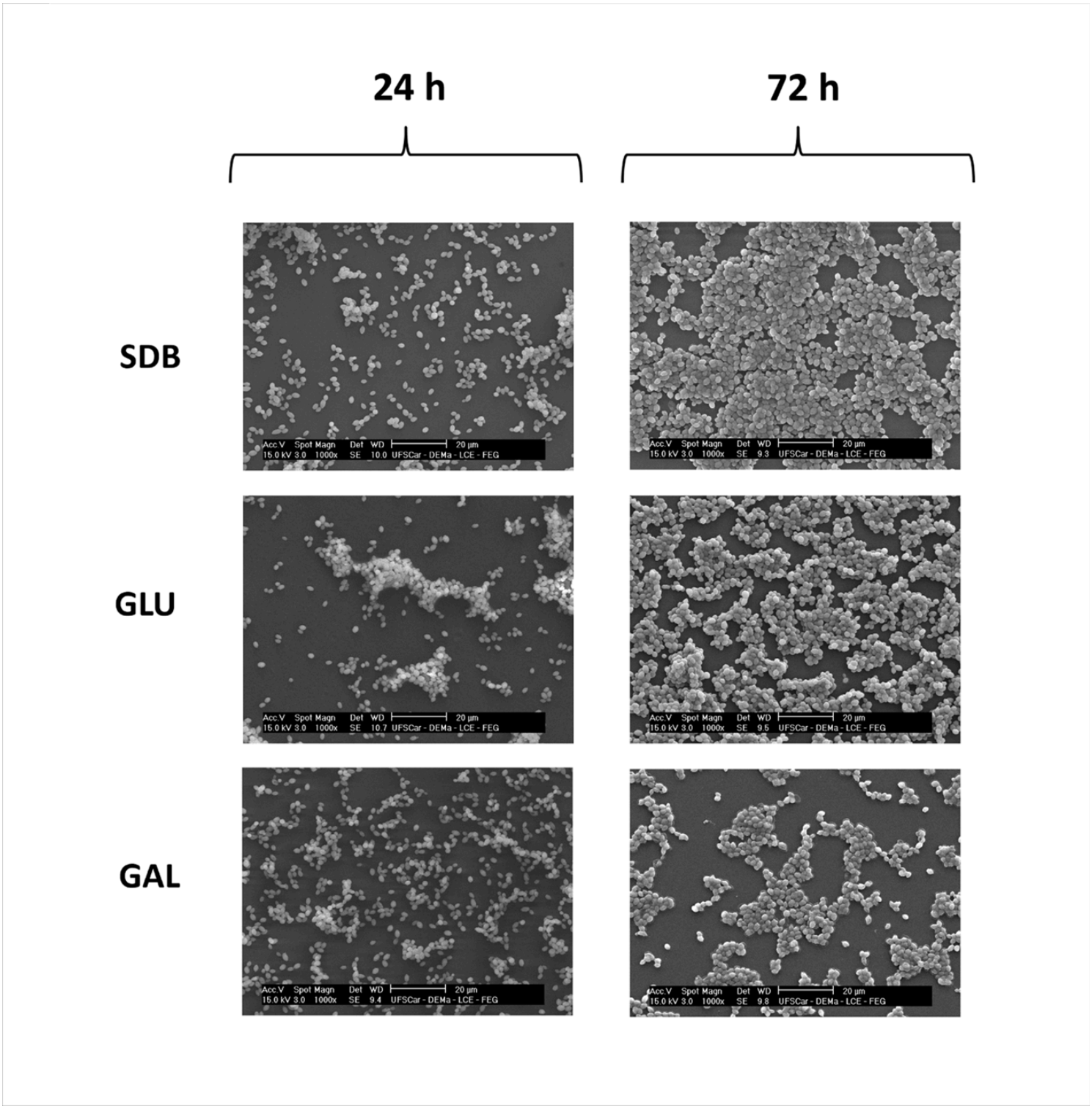


Figure 4

Scanning electron microscopy images of *Nakaseomyces glabratus* biofilms formed under static conditions for 24 or 72 hours in Sabouraud dextrose broth (SDB), SDB supplemented with 500 mmol/L glucose (GLU), and SDB supplemented with 500 mmol/L galactose (GAL). (Magnification: ×1000; scale bar = 10.0 µm).

Alt text: Scanning electron micrographs of *Nakaseomyces glabratus* biofilms formed for 24 and 72 h under static conditions in SDB, GLU, and GAL media, showing clusters of yeast cells and sparse extracellular matrix. Scale bar represents 10 μm.