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Short communication

Fluorescence-based assessment of viability and cell enumeration of the probiotic yeast *Saccharomyces boulardii*

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Introduction

Probiotics are live microorganisms that provide health benefits when administered in adequate amounts (Hill et al., 2014). *Saccharomyces boulardii (S. boulardii)* is one of the most widely used probiotic yeasts due to its antibiotic resistance and tolerance to the gastric environment (Pais et al., 2020). As probiotic efficacy depends on cell viability, reliable monitoring is essential, particularly in lyophilized products requiring rehydration. Although the conventional plate-count (CFU) method is well established, it is time-consuming and cannot detect viable but non-culturable cells (Chiron et al., 2018). In contrast, acridine orange/propidium iodide (AO/PI) fluorescence staining enables rapid differentiation between viable and non-viable cells based on membrane integrity (Hussain et al., 2019). Therefore, this study aimed to establish an automated AO/PI fluorescence method for simultaneous determination of total cell concentration and viability of *S. boulardii* after rehydration and to compare its performance with the conventional CFU method.

Materials and methods

A commercial lyophilized preparation of *S. boulardii* (1.5×10^{10} CFU/g), kindly donated by Green Agro (North Macedonia), was used as the test material. YPD broth and agar (GRISP, Portugal) were used for cultivation, while acridine orange/propidium iodide (AO/PI, Logos Biosystems, South Korea) served as fluorescent stains.

Two grams of lyophilized *S. boulardii* were rehydrated and activated in YPD broth for 2 days under controlled temperature to obtain a metabolically active suspension (Pais et al., 2020). Serial ten-fold dilutions were prepared in peptone water. To evaluate method performance, part of the culture was heat-killed and mixed with live cells to obtain reference suspensions containing 25%, 50%, 75%, and 100% viable cells.

Total cell concentration and viability were determined by AO/PI dual-fluorescence staining using a LUNA-FL™ automated fluorescence cell counter (Logos Biosystems, South Korea). Agreement between measured and nominal viability was assessed by linear regression (R^2) and root-

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mean-square error (RMSE). For reference enumeration, appropriate dilutions were plated (0.5 mL) on YPD agar, incubated, and colonies counted to calculate CFU/mL using the colony count, plated volume, and dilution factor (Chiron et al., 2018).

Results and discussion

Rehydration in YPD broth produced metabolically active suspensions with total cell concentrations of 10^6–10^7 cells/mL, within the reliable counting range. Comparable cell concentrations were obtained by AO/PI fluorescence staining, while the fully live control showed 97–99% viability, confirming the high native viability of the rehydrated culture.

Method accuracy was evaluated using mixtures of live and heat-killed cells with defined viabilities. Within the optimal counting range, measured viability increased linearly with the live-cell fraction and closely matched the nominal values. The 25%, 50%, and 75% standards were recovered as 24.8%, 50.6%, and 76.9%, respectively. Among the five dilutions tested, the best agreement was obtained at 1:10 and 1:10^7 (R^2 = 0.97–0.99, RMSE ≈ 3–5 percentage points), whereas performance deteriorated outside the optimal density range, highlighting the importance of appropriate dilution and green/red gating.

Plate counting provided an independent validation. Colony counts increased monotonically with the live-cell fraction, ranging from 2.6×10^6 to 1.6×10^7 CFU/mL across the 25%, 50%, 75%, and 100% live samples, and showed a strong linear relationship with nominal viability (R^2 = 0.97). Based on the fluorescence results, plating was performed at the 1:10^7 dilution, as the 1:10 dilution produced excessively concentrated, uncountable plates. Relative to the fully viable control, the corresponding viabilities were 16%, 54%, and 85% (RMSE = 6.9 percentage points). The comparable linearity obtained by both membrane-integrity staining and colony formation supports the validity of the fluorescence-based method (Chiron et al., 2018).

For absolute enumeration, AO/PI staining yielded approximately 6.44×10^6 cells/mL, while agar plate counting gave approximately 1.6×10^7 CFU/mL. Although not identical, the results were of the same order of magnitude. Importantly, fluorescence staining provided results within minutes, whereas agar plate counting required several days for colony growth.

Conclusion

An automated AO/PI fluorescence method was successfully established for rapid determination of *S. boulardii* viability and total cell concentration after rehydration. The method showed good agreement with conventional plate counting while substantially reducing analysis time, supporting its use for routine probiotic viability assessment.

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