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Development of a High-Cell-Density Production Process for a Biotherapeutic Yeast, *Saccharomyces cerevisiae* var. *boulardii*, for Use as a Human Probiotic

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Abstract: *Saccharomyces cerevisiae* var. *boulardii* is a probiotic yeast widely recognized for its ability to enhance gut health and modulate a host's microbiome. However, there are limited data on its large-scale cultivation in stirred tank bioreactors and subsequent downstream processing into a functional probiotic product. Different recipe formulations were evaluated and the recipe with the highest biomass yield and lowest process time was selected. Once the optimised batch was validated in the replicate batches, the statistical analysis indicated a high level of reproducibility, with low variability across key performance indicators such as biomass concentration (unit), CFU production (CFU.mL^{-1}), and substrate utilization efficiency (g.g^{-1}). The mean growth age in the bioreactor was 25.33 ± 1.16 h, with a CV of 4.56%, indicating minimal deviation between batches. Similarly, the final viable concentration exhibited a mean of 1.46 × 10^{8} CFU.mL^{-1} with a CV of 11.68%, remaining within an acceptable range for biological processes, while the final biomass concentration had the lowest variability (CV of 3.94%) and a 95% CI of 12.134–13.266 g.L^{-1}, highlighting the accuracy and consistency of the process. Productivity indicators, including cell productivity (growth time—biomass) and YPP (biomass), maintained low CV values (3.933% and 3.389%, respectively), reinforcing process efficiency and stability. The overlapping 95% confidence intervals across batches further confirmed that no statistically significant deviations existed, ensuring minimal batch-to-batch variability, and validating the scalability and robustness of the fermentation process. These findings provide strong evidence for the feasibility of large-scale probiotic yeast production that meets industrial production standards. The final freeze-dried product retained an 81% viability post-exposure to simulated gastrointestinal conditions, meeting WHO probiotic viability standards. These findings establish a scalable, optimized process for probiotic yeast production, with potential applications in biopharmaceutical manufacturing and functional food development, as confirmed by the techno-economic evaluations performed using SuperPro Designer®.

Keywords: *Saccharomyces cerevisiae* var. *boulardii*; probiotics; production; formulation; simulated gastric testing; techno-economic assessment

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1. Introduction

The extremely complex, diverse, and dynamic population of microorganisms in the intestine is collectively known as the gastrointestinal or gut microbiota [1]. A disruption of the gastric equilibrium due to negative manipulation or an alteration of the gut microbiota results in an adverse intestinal microbiota disbalance termed intestinal dysbiosis [2,3].

Intestinal dysbiosis consequently triggers the proliferation of pathogenic microbes [4] or inflammatory effects leading to clinical disorders such as gastrointestinal, cardiovascular, autoimmune, and metabolic diseases [5,6]. Attempts to ameliorate the health status of individuals affected by intestinal dysbiosis include administering probiotic supplements in appropriate quantities to modulate the diversity or properties of the indigenous intestinal flora [7,8]. As defined by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), probiotic adjuncts are "live microorganisms, when administered in sufficient amounts provide a health benefit to the host" [9].

Yeast probiotics have less popularity than their bacterial counterparts, however, their enticing properties enhance their prospects as successful probiotic candidates. Yeast probiotics have a higher chance of survival in the stomach as they can tolerate bile salt stress conditions, adhere to epithelial cells, possess resistance against gastrointestinal conditions and various antibiotics, and possess antimicrobial activity against pathogens [10,11]. *Saccharomyces* is the only yeast genus that demonstrated effective probiotic qualities in double-blind clinical studies [12], with *S. cerevisiae* [13–15] and *S. cerevisiae* var. *boulardii* [10,16] being the only probiotic yeasts with a Generally Recognised as Safe (GRAS) status and that are available commercially for human consumption. The notable important physiological characteristics of *S. cerevisiae* var. *boulardii* include its innate thermo- and acid tolerance as well as an ability to grow optimally at 37 °C and survive at pH 2.5, which represent the physiological conditions of the human digestive tract [17,18]. *S. cerevisiae* var. *boulardii* demonstrated clinical significance as an efficacious biotherapeutic agent against ulcerative colitis [19,20], in the management of the Crohn's disease activity index [21], in treating inflammatory bowel disease [22] and chronic diarrhoea [23,24], in reducing the inflammation and dysfunction of the gastrointestinal tract induced by antibiotics and medication [20,25], and in its antagonistic fungal [26] and bacterial [27] effects.

Undoubtedly, there is growing scientific evidence supported by extensive mechanistic research and promising clinical studies that *S. cerevisiae* var. *boulardii* probiotics provide vast potential to improve human health. This might create challenges for probiotic industries wherein various strategies need to be explored to achieve high-cell-density cultivation technologies. Bioreactor cultivation has been successfully employed for the high-cell-density cultivation of probiotic yeasts such as *Kluyveromyces lactis* and *S. cerevisiae* in 16 L stainless steel [28], and a 5 L stirred tank bioreactor, respectively [29]. Reports have also outlined the cultivation of *S. cerevisiae* var. *boulardii* in a 3 L stirred tank bioreactor, a 10 L Biostat bioreactor [30], and a 16 L stirred tank bioreactor [31].

This study aimed to develop an efficient bioprocess for high-cell-density production of *S. cerevisiae* var. *boulardii* in stirred tank bioreactors at a 30 L scale. Firstly, media recipes were designed and evaluated using batch and fed-batch cultivation strategies to determine the optimal media and cultivation conditions to produce high cell densities. The yeast biomass was recovered via centrifugation, the pellet was freeze-dried with lyoprotectant, and the viability of the freeze-dried *S. cerevisiae* var. *boulardii* under simulated gastric fluid and simulated intestinal fluid conditions was assessed. Lastly, a techno-economic feasibility assessment of the probiotic production process was conducted to determine the cost of manufacturing during commercial production (1000 L or 1 m^3) using SuperPro Designer® and inputs from the lab scale process demonstration (30 L). These findings will provide insight into developing innovative high density biomass production strategies and tailored production processes for *S. cerevisiae* var. *boulardii* and other probiotic candidates, as well form justifications for localising the production of bio-based products such as probiotics on the African continent.

2. Materials and Methods

2.1. Yeast Strain Identification and Preservation

S. cerevisiae (Accession no. KY105187.1) was isolated from a probiotic product supplied to the CSIR. The stress resistance capability of this *S. cerevisiae* strain was marked by its ability to proliferate on yeast-peptone-dextrose (YPD) (Merck Darmstadt, Germany) broth adjusted to pH 3 and incubated at 37 °C. This ability to grow under these acidic conditions differentiates the *S. cerevisiae* var. *boulardii* from the *S. cerevisiae* strain. The strain was cryopreserved as 50% ($v.v^{-1}$) glycerol stocks in sterile 2 mL Cryo.S™ tubes (Greiner Bio-one, Frickenhausen, Germany) and stored at −80 °C (Forma 8800 Series, Thermo Scientific, Waltham, MA, USA) for further use.

2.2. Cultivation for High-Cell-Density Production

2.2.1. *S. cerevisiae* var. *boulardii* Inoculum Preparation

A single 2 mL cryovial was inoculated into a Fernbach flask containing 700 mL of YPD broth (Merck Darmstadt, Germany) and incubated at 37 °C in a rotary platform shaker (New Brunswick Scientific, Edison, NJ, USA), agitated at 180 rotation per minute (rpm) for 18 h. Once the transfer optical density of 4 was achieved at 600 nm (OD_{600}) (Pharo 300 Spectroquant®; Merck Milipore), the flasks were microscopically assessed for their monoseptic status under 1000× magnification, using an Olympus BX40 microscope (Olympus, Tokyo, Japan).

2.2.2. 30 L Bioreactor Cultivation

Variations of a semi-defined medium were assessed to produce *S. cerevisiae* var. *boulardii*. The media consisted of yeast extract (1–4%), glucose (1–5%), $NaNO_3$ (0.1–0.5%), KH_2PO_4 (0.4–0.8%), and $MgSO_4.7H_2O$ (0.4–0.8%) at different supplementation levels. The 30 L Biostat reactor (Sartorius BBI Systems, Melsungen, Germany) was operated at a working volume of 24.3 L. A trace element solution (48.6 mL) composed of ($Unit.L^{-1}$) 1 L distilled H_2O, 20.1 mL Na-EDTA, 16.7 mg $FeCl_3.6H_2O$, 0.13 mg $ZnCl_2$, 0.18 mg $CoCl_2.6H_2O$, 0.13 mg $Na_2MoO_4.2H_2O$, 0.5 mg $CaCl_2$, 0.16 mg $CuSO_4.5H_2O$, 0.28 mg H_3BO_3, and 0.11 mg $MnCl_2.2H_2O$ was used. A vitamin solution (100 mL) composed of ($Unit.L^{-1}$) 1 L distilled H_2O, 9.52 mg $NaH_2PO_4.2H_2O$, 1.2 mg $Na_2HPO_4.7H_2O$, 9.52 mg meso-inositol, 9.52 mg nicotinic acid, 9.52 mg biotin, and 9.52 mg thiamine HCl was filter sterilised into the transfer vessel and transferred into the bioreactor using a peristaltic pump (Watson-Marlow, Falmouth, UK) post sterilisation.

The contents of the reactor were sterilised in situ at 121 °C for 45 min. A 42% $m.m^{-1}$ TSAI glucose solution was prepared and autoclaved separately at 121 °C for 60 min (Eins Sci, Hospi Sterilizers, Johannesburg, South Africa) and added aseptically using a peristaltic pump. Each bioreactor was inoculated using a single inoculum flask of *S. cerevisiae* var. *boulardii*. The fermentation was maintained at 37 °C with the stirrer speed set at 100 rpm. Aeration was set at 1 $v.v^{-1}.m^{-1}$ and the dissolved oxygen was maintained at >30% saturation. A pH of 5.5 was maintained using either 2.5 M HCl or 2.5 M NaOH and antifoam (25 % $v.v^{-1}$) (Durapol 3000, ProtonChem, Durban, South Africa) was added at a concentration of 1 $mL.L^{-1}$ when required. The fermenter was operated with a back pressure of 500 mBar. Unless stated otherwise, all reactor cultivations were maintained at these process parameters. A glucose feed was initiated when the residual glucose concentration in the fermenter reached ~1 to 5 $g.L^{-1}$, and the feed rate was adjusted incrementally to maintain residual glucose levels at this target concentration. Feeding was stopped when no further increase in OD_{600} (stationary phase) or cell concentration was observed. The cell material was then harvested into pre-sterilised vessels.

2.2.3. Validation of Cultivation Process at 30 L Scale

Based on the performance data obtained, the best performing recipe was selected for further studies based on the highest productivity of the biomass and viable cell concentrations achieved. The cultivations were performed in triplicate under the same process control conditions listed above (n = 3).

2.3. Sampling and Data Analysis

The fermentation samples were harvested every 2 h for analysis under aseptic conditions. Glucose concentrations ($g.L^{-1}$) were determined using an Accutrend® alpha glucometer (Boehringer Mannheim, Germany). The total cell concentration ($cells.mL^{-1}$) was determined by enumerating the cells under an Olympus BX40 microscope (Olympus, Japan) using a Thoma counting slide (Hawksley and Sons, London, UK). Biomass production ($g.L^{-1}$) was determined by aliquoting 2 mL of a sample into a pre-weighed 2 mL microcentrifuge tube (Eppendorf, Hamburg, Germany) followed by centrifugation (Biofuge pico, Baden-Württemberg, Germany)) for 10 min, at 4 °C, and at 13,000 rpm (n = 4). The supernatant was discarded, and the pellet was resuspended in 750 µL 0.1 M HCl and centrifuged for 10 min, at 4 °C, and at 13000 rpm. The pellet was washed with 750 µL ultrapure water (Milli-Q® Direct, Merck, Germany) and centrifuged for a further 5 min, at 4 °C and at 13,000 rpm. Thereafter, the pellet was oven-dried at 105 °C for ± 24 h. Once dry, the tubes were placed in a desiccator for ± 12 h and weighed using an analytical balance (Mettler Toledo AE 200, Woonsocket, RI, USA). The final viable cell concentration was determined using a standard plate count method by serially diluting the samples and spread-plating them onto YPD agar (Merck, Germany) (n = 4), followed by incubation at 37 °C for 48 h. Following incubation, colony forming units (CFUs) were enumerated using a colony counter (Bibby, Stuart scientific, Stone, Staffordshire, UK).

The statistical method used to compare the batch recipes was a one-way ANOVA (SPSS V29). This was used to compare the performance parameters for cell concentration, viable cell concentration, biomass concentration, productivity, yield on protein, and yield on sugar across the four different recipes. This statistical approach determined whether significant differences existed among the recipes, or not.

Determination of Key Process Indicators and Statistical Analysis

The results obtained from the fermentation batches (n = 3) were used to assess the reproducibility and robustness of the production process using statistical tools. The three batches were compared using SPSS V29 (IBM, CORP, Armonk, NY, USA). Descriptive statistics including the mean, standard deviation (SD), coefficient of variation (CV%), and 95% confidence intervals (CIs) were determined. The results assessed key performance indicators such as biomass concentration, CFU production, cell productivity, as well as the yields on substrates added. These indicators were used to compare batch-to-batch variability to determine the consistency of the fermentation process.

The key process parameters used to assess the performance of the fermentations were determined using biomass concentration ($g.L^{-1}$). The yield of the product produced per unit of carbohydrate (YP_S), or protein (YP_P) consumed, and productivity were calculated using the following equations:

$$YP_S(g.g^{-1}) = \frac{\text{Biomass}_{\text{final}} \times \text{volume}_{\text{final}}\ (L)}{\text{Total TSAI added}\ \left(g.L^{-1}\right)}$$

$$YP_P\left(g.g^{-1}\right) = \frac{\text{Biomass} \times \text{volume}_{\text{final}}\ (L)}{\text{Total protein}\ \left(g.L^{-1}\right)}$$

$$\text{Biomass productivity}\ (g.L^{-1}.h^{-1}) = \frac{\text{Biomass}_{\text{final}}}{\text{volume}_{\text{final}}\ (L) \times \text{process age}\ (h)}$$

2.4. Downstream Processing

2.4.1. Cell Harvesting Post Separation and Cell Separation

Upon termination of the bioreactor cultivation, the *S. cerevisiae* var. *boulardii* cell material from each fermenter was harvested into separate pre-sterilised storage vessels. Solid–liquid separation was performed using a disk stack centrifuge BRPX 207 (Westfalia, SA1, GEA, Oelde, Germany) using a single pass process. The centrifuge was operated at a constant speed of 8500 rpm and the bowl pressure was maintained at 100 kPa. Fermentation broth was fed into the centrifuge at a flow rate of 52 $L.h^{-1}$ with a de-sludge time of 20–25 min. The harvested material, supernatant, and pellet were serially diluted and plated on a YPD agar. The plates were incubated for 24–48 h at 37 °C and colony forming units (CFUs) were enumerated and used to determine viable cell recovery during the process unit operation.

2.4.2. Lyophilization

The *S. cerevisiae* var. *boulardii* biomass obtained post centrifugation was mixed with lyoprotectant LySol (Protonchem, South Africa) at a ratio of 1:1, frozen in fast-freeze flasks at −80 °C for 12 to 15 h (Forma 8800 series, Thermo Scientific, USA), and freeze-dried (VirTis BenchTop K-Series- SP Industries, Inc., Warminster, PA, USA) for 36 h. The viable cell population of *S. cerevisiae* var. *boulardii* prior to and post freeze-drying was determined by spread-plating serially diluted samples on a YPD agar and incubating them for 24–48 h at 37 °C. The CFUs were enumerated and used to calculate cell recovery.

2.5. Determining Viability of Freeze-Dried S. cerevisiae var. boulardii in Simulated Gastrointestinal Fluid Conditions

2.5.1. Assessment of Freeze-Dried Material Pre-Exposure

Total cell concentration ($cells.mL^{-1}$) post freeze-drying was determined by enumerating the cells using an Olympus BX40 microscope (Olympus, Japan) and using a Thoma counting slide (Hawksley and Sons, London, UK). This analysis was performed to determine the dilution series. Viable cell enumeration was conducted as previously described.

2.5.2. Preparation of Simulated Gastric Fluid and Simulated Intestinal Fluid Solutions

The simulated gastric fluid (SGF) solution was prepared by modifying the methods described by [32]. To 1000 mL of saline (0.5% $m.v^{-1}$), 3 g of pepsin (Sigma Aldrich, St. Louis, MO, USA) was added, and the pH was adjusted to between 2 and 3 using 12 N HCl. The simulated intestinal fluid (SIF) solution was prepared by modifying the methods described by [32,33]. Into a 1000 mL beaker, 6.8 g of KH_2PO_4 was added followed by 250 mL of distilled water and this was mixed thoroughly. Thereafter, 77 mL of 0.2 N NaOH was mixed in and a further 300 mL of distilled water was added. The solution was adjusted to a pH of 7 using either 0.2 N NaOH or 0.2 N HCL. After the pH adjustment, 1 g of pancreatin (Sigma Aldrich, Missouri USA) was added and the solution was made up to a final volume of 1 L.

2.5.3. Cultivation of Freeze-Dried *S. cerevisiae* var. *boulardii* Under SGF Conditions to Assess Viability

In a 15 mL falcon tube (Corning, New York, NY, USA), 9 mL of a filter-sterilised (0.22 µm Minisart® filter; Sartorius Stedim Biotech, Gottingen, Germany) SGF solution was added to 1 g of freeze-dried *S. cerevisiae* var. *boulardii*. The solution was mixed for 30 s to obtain complete dispersion and incubated at 37 °C on a rotary platform shaker incubator (New Brunswick Scientific, USA) at 100 rpm for 2 h. This step was performed to determine the product's ability to survive and proliferate in the gastric environment. Upon inoculation of the freeze-dried *S. cerevisiae* var. *boulardii*, a 1 mL sample was withdrawn immediately, designated T000, and thereafter another 1 mL sample was taken after two hours of incubation, designated T001. Viable cell enumeration was conducted as previously described.

2.5.4. Cultivation and Viability of Freeze-Dried *S. cerevisiae* var. *boulardii* Under SIF Conditions

After the 2 h incubation period, the tubes were centrifuged (Allegra, X-22R, Fullerton, CA, USA) for 10 min, at 4500 rpm. The supernatant was discarded, and the remaining pellet was washed with 4 mL of a filter-sterilised SIF solution and re-centrifuged under the same conditions. The supernatant was discarded, and the pellet was resuspended in 9 mL of a filter-sterilised SIF solution. The tubes were incubated at 37 °C on a rotary platform shaker (New Brunswick Scientific, USA) at 100 rpm. A 1 mL sample was taken immediately after the product was added to the tubes containing SIF, designated T002. The test samples were incubated further on a rotary platform shaker at 37 °C and 100 rpm for 3 h (T003) and 6 h (T004), respectively, and enumerated as previously described. This step was performed to determine the product's ability to survive and proliferate in the intestine after being exposed to the gastric environment.

2.6. Techno-Economic Feasibility Assessment

To elucidate the feasibility of the production process, a SuperPro Designer® v13 was used to determine the cost of manufacturing a freeze-dried *S. cerevisiae* var. *boulardii* product. Both the upstream and downstream unit operations were detailed, using the data and process flow sheets compiled using the 30 L investigations and simulations, and were created to determine the cost implications of a $1m^3$ facility using the raw materials available for use in South Africa.

3. Results and Discussion

3.1. Production of S. cerevisiae var. boulardii in 30 L Bioreactors

Four growth medium formulations were tested, and the performance of each formulation was assessed based on the total and viable cell concentrations as well as the biomass concentration. Recipe 3 demonstrated the best process performance based on total and viable cell concentration, biomass production, as well as productivity based on the biomass and viable cell concentrations (Figure 1a–c). Due to the intended application of the biomass as living probiotic cells, the viable cell concentration data could be used to ascertain process performance using the different recipes tested. However, limited comparative information was available in the literature; hence, biomass concentration was used. The biomass concentration obtained during the cultivation of *S. cerevisiae* var. *boulardii* using recipe 3, was 50% higher than the performance reported by [31].

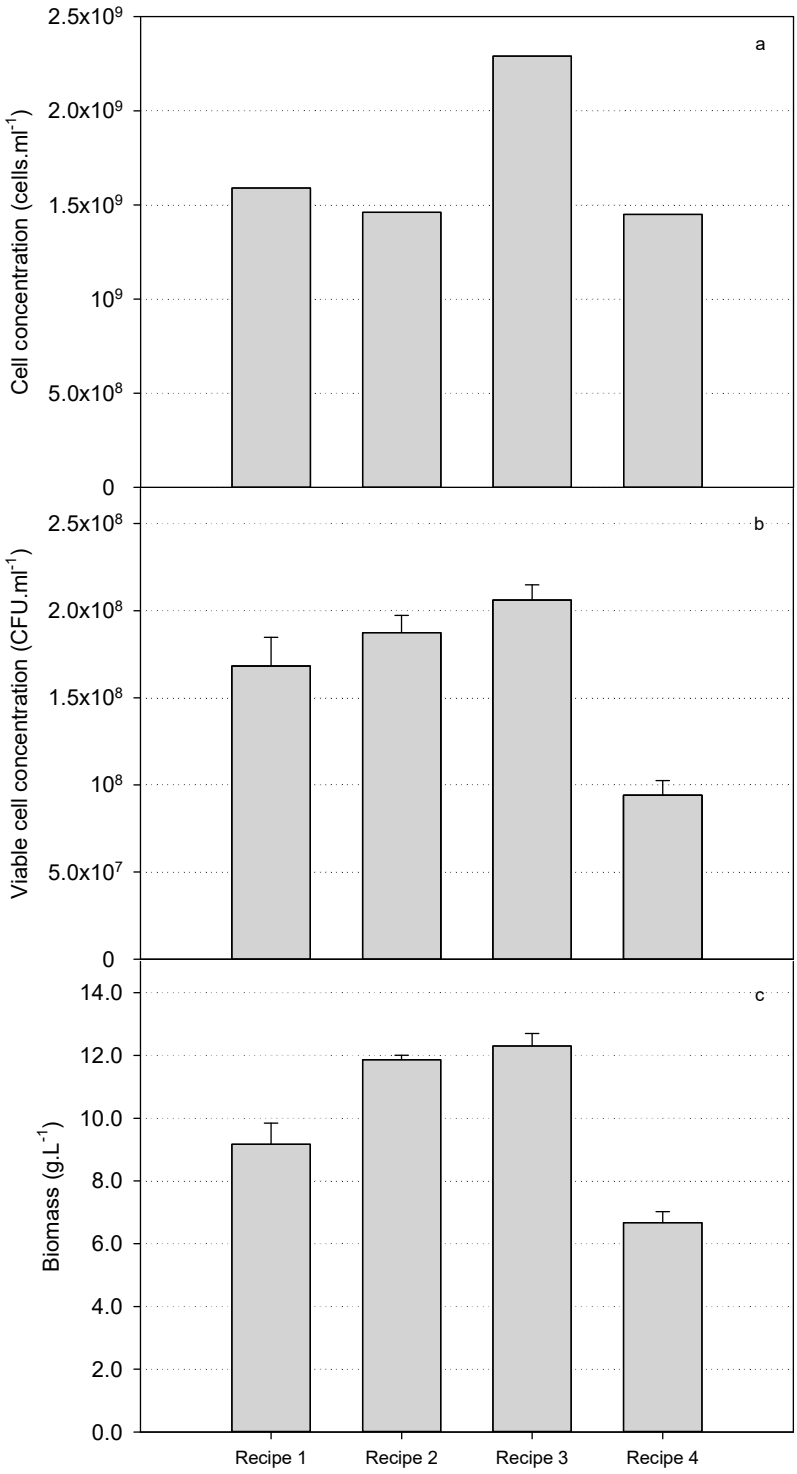


Figure 1. Production values obtained during the cultivation of *S. cerevisiae* var. *boulardii*, in 30 L stirred tank bioreactors, detailing total cell concentration (**a**), viable cell concentration (n = 4) (**b**), and biomass concentration (n = 4) (**c**).

Upon an assessment of the YPP and YPS indicators determined using the amount of biomass produced, it was noted that recipe 3 was outperformed by test recipe 2 (Figure 2a,b). This was a consequence of recipe 2 having a lower yeast extract supplementation level as well as having the lowest consumption of carbohydrates during the fed-batch process. As a result, this recipe (#2) demonstrated the best yield on both protein and carbohydrates of those tested in this study (Figure 2a,b). This will result in a lower raw material cost of production. In typical bioprocesses, process time is generally the most pertinent parameter

as space time yield is a critical factor in bioprocess operations. Therefore, highest productivity was used as the over-riding selection criterion to select the best growth medium formulation to cultivate *S. cerevisiae* var. *boulardii* in stirred tank reactors. The error bars representing the standard deviation (n = 4), shown in Figures 1 and 2, provide clarity on the reproducibility and reliability of the results obtained from the recipes that were tested.

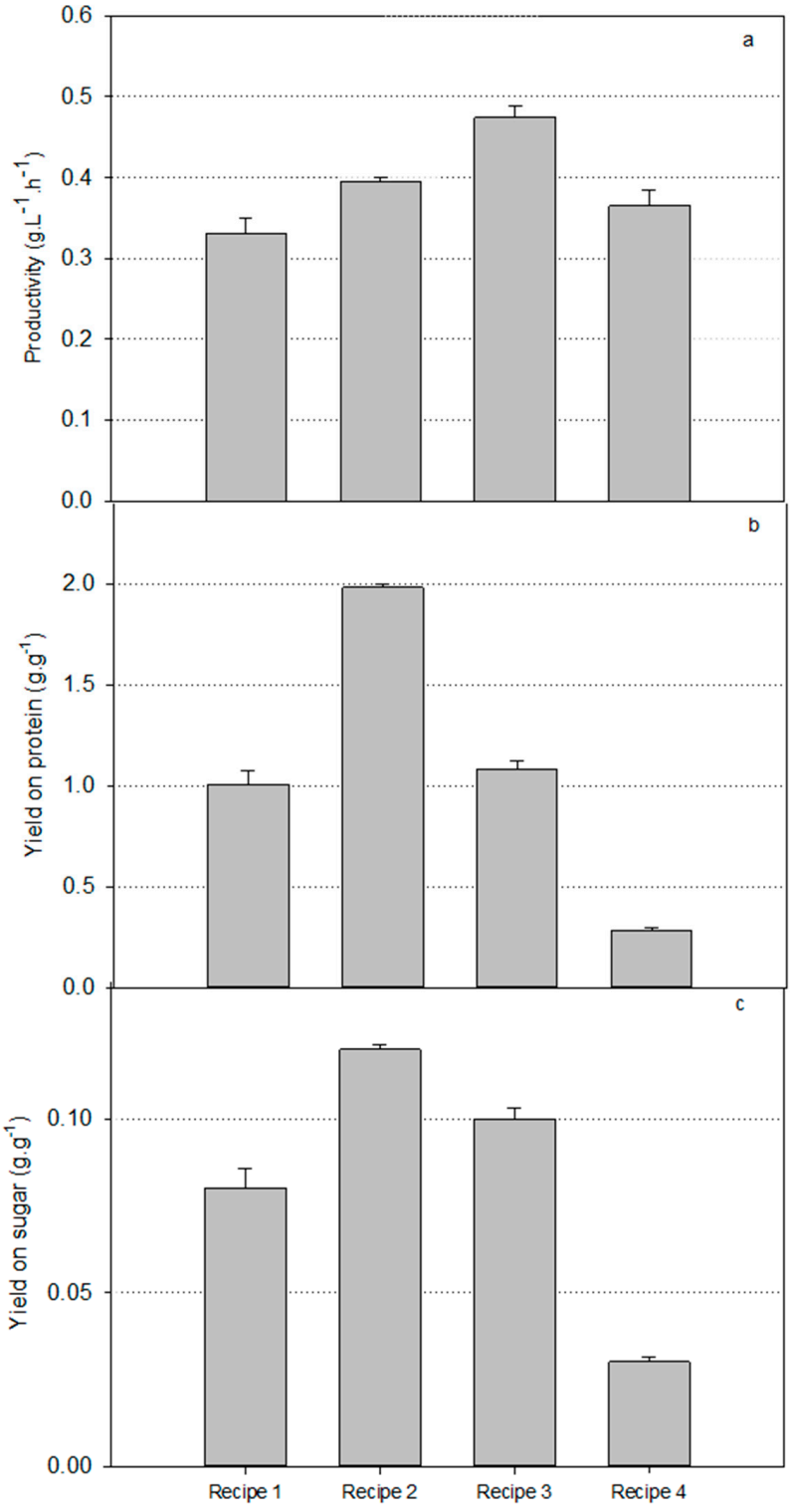


Figure 2. Key process indicators obtained during the cultivation of *S. cerevisiae* var. *boulardii*, in 30 L stirred tank bioreactors detailing yield of biomass on protein (**a**), yield of biomass on carbohydrate supplemented (**b**), and biomass productivity (**c**) (n = 4).

Consequently, recipe 3 was selected for all further research and development activities (Figure 2c). A comprehensive comparison of process performance could not be conducted, as little to no reports on the production performance of *S. cerevisiae* var. *boulardii* are available in the literature sources. Hence, this study presents a basis for future studies and can be used as a benchmark for other human probiotic production processes. Overall, the statistical significance across all measurements confirmed that the differences between the fermentation recipes were significant (p-values < 0.001).

3.2. Validation of a Fed-Batch Fermentation Process for the Cultivation of S. cerevisiae var. boulardii

The statistical analysis in Table 1 confirms that the fermentation process was highly reproducible, with low variability in key performance indicators such as biomass concentration, CFU production, and substrate utilization efficiency. The growth age in the fermenter was 30 h based on the maximum biomass concentration, correlating to a final viable cell concentration of 1.46×10^8 ($\pm 1.67 \times 10^8$) CFU.mL^{-1}. In addition, biomass concentration had the lowest variability (CV of 3.40%) and a 95% CI of 12.13–13.27 g.L^{-1}, highlighting the repeatability of the process.

Table 1. Process indicators obtained during the replicate cultivations of *S. cerevisiae var. boulardii* in 30 L stirred tank bioreactors (n = 3).

Measured Variable	Unit	Mean	Std Dev	Coefficient of Variance	95% CI Lower	95% CI Upper
Growth age in fermenter	h	25.33	1.16	4.56	24.03	26.64
Final biomass concentration	g.L^{-1}	12.70	0.50	3.94	12.13	13.27
YPS	g.g^{-1}	0.20	0.01	4.47	0.19	0.21
YPP	g.g^{-1}	23.31	0.79	3.39	22.42	24.20
Cell Productivity	g.L^{-1}.h^{-1}	0.42	0.02	3.93	0.40	0.44

Productivity indicators, including cell productivity and YPP (biomass), maintained low CV values (3.93% and 3.39%, respectively), reinforcing process efficiency and robustness. The minimal variation observed, supported by the low CV% values (<14%) and overlapping confidence intervals, demonstrates that the batches followed consistent and predictable trends, by ensuring that the process was well controlled. Thus, this process was deemed suitable for large-scale application. In our experience, we have noted that due to the biological nature of microbial cultivation processes, process variations in terms of performance may occur anywhere in the range of 10 to 30%. Therefore, the acceptable range of variation must be decided upon by the end user of the technology depending on the performance criteria required. The low variation observed in this study can be attributed to the characterization of the inoculum process, as well as the tight operator control exercised during the replicate cultivations. The findings from this study confirm the feasibility of the process for large-scale implementation, with high reproducibility and consistent fermentation performance.

3.3. Recovery After Centrifugation and Freeze-Drying

Post cultivation, approximately 27 L of *S. cerevisiae* var. *boulardii* cell material was harvested from the reactors. The average recovery rate across the separation process was 94.94 ± 6.26% with associated cell losses in the supernatant of <4% for the replicate batches. A single centrifugation pass was used to recover *S. cerevisiae* var. *boulardii* biomass and no reconstitution step was required for this stage of the process technology. This eliminated the need for additional centrifugation steps and a reconstitution of the pellet

into a suitable buffer which adds to the cost of production, in terms of manpower and consumable costs [34]. An ideal solid–liquid separation step is generally designed to occur at a fast rate and have high recovery efficiencies. In addition, the selection of the process unit operation should also require low capital investment which can operate at a minimal life cycle cost [34].

Post centrifugation, the cells were frozen prior to being freeze-dried. By including a commercial lyoprotectant, Lysol, an average freeze-drying recovery rate of 78.21% was obtained through the drying unit operation. In many instances, the downstream processing (DSP) costs associated with recovering cells from a broth medium can be in the region of 15 to 70% of the total manufacturing costs [35]. Therefore, it is imperative to ensure that astute decisions are made regarding the selection of DSP steps, to obtain maximum cell viability such that a techno-economically feasible cost of production is achieved.

3.4. Viability of S. cerevisiae var. boulardii After Exposure to Simulated Gastrointestinal Environment Conditions

Once a freeze-dried product was generated, the survivability of the product was assessed using simulated gastrointestinal conditions. According to the International Scientific Association for Probiotics and Prebiotics, tolerance against the gastrointestinal tract conditions is a precondition for a microorganism to be eligible as a probiotic. To qualify, the probiotic must survive after being subjected to gastric fluid; moreover, it is integral that the probiotic maintain viability in the gut and exert its full function [36]. The SGF and SIF assessment aimed to elucidate the viability of freeze-dried *S. cerevisiae* var. *boulardii* after being exposed to the conditions exhibited in the gastrointestinal tract of a human host. The use of an acidic pH (2 to 3) and the addition of pepsin is important as it accurately mimics the human gastric conditions. It also provides a realistic assessment when evaluating the survival and viability of prospective probiotics in the gastric environment [11]. In the present study, the starting cell concentration of the freeze-dried *S. cerevisiae* var. *boulardii* product was 2.11×10^9 ($\pm 1.80 \times 10^8$) cells.mL^{-1} (Figure 3). This starting material would theoretically represent the probiotic prior to ingestion. After the addition of the probiotic to the SGF there was an increase in the cell concentration to 2.93×10^9 cells.mL^{-1}. After two hours of exposure in the SGF, the cell concentration decreased to 1.37×10^9 cells.mL^{-1}, indicating that only 35% of the *S. cerevisiae* var. *boulardii* cells were viable post exposure to the SGF environment. The extremely acidic environment of the stomach (pH 2 to 3) in combination with the presence of the digestive enzyme pepsin, poses a significant inhibitory challenge to probiotics. Some microorganisms, including yeasts, possess the capability to survive and grow in an acidic medium. The underlying mechanism by which a yeast cell can survive at a low pH is through a modification of the cell wall [37,38]. When yeast cells are in the presence of strong inorganic acids or a low pH, such as hydrochloric acid in the stomach, a cell wall integrity pathway is activated [39,40]. This leads to the formation of various carbohydrate polymers used for remodelling the cell wall [41]. Alternatively, a general stress response pathway is initiated [38] as observed for *Candida glabrata*, whereby the membrane lipid composition is adjusted by mediator subunits, leading to an increased acid tolerance [42]. A report by [43] states that *S. cerevisiae* acquires acid tolerance by altering the sterol composition in the cell wall and reducing iron uptake.

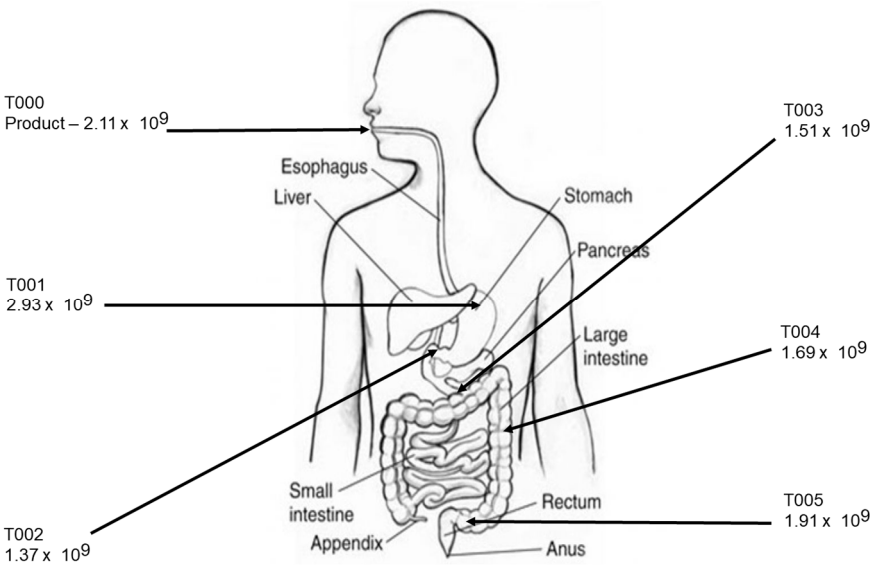


Figure 3. An illustration detailing the functionality of the probiotic, *S. cerevisiae var boulardii*, upon transit through a simulated digestive environment.

After three hours of exposure to SIF, a cell concentration of 1.51×10^9 cells.mL^{-1} was noted, equating to a survivability rate of 71% post-ingestion (Figure 3). After an additional six hours of exposure to SIF, the final cell concentration was 1.69×10^9 cells.mL^{-1}. Overall, from the starting material (pre-ingestion) to the material after exposure to SGF and SIF conditions (post-digestion), a noteworthy survivability rate of 80.09% was observed. This infers that *S. cerevisiae* var. *boulardii* maintained viability as it transitioned through the simulated gastrointestinal passage, despite the initial drop in viability post exposure to gastric conditions. To confer health benefits to the human host, probiotics should survive transit through the acidic conditions in the stomach whilst reaching the intestine in large amounts to efficiently colonise and proliferate [44]. The WHO's regulation recommends that functional products or foods claiming to have beneficial probiotic preparations, should contain 10^6 to 10^8 viable probiotic cells (colony forming units) per gram (CFU/g) [9]. The International Dairy Federation (IDF) recommends a minimum number of 10^6 viable probiotic cells per millilitre (CFU/mL) of product at the time of consumption [45]. The final cell concentration of 10^9 noted herein aligns with the WHO's recommendation for functional probiotics with beneficial qualities. The 80% viability of *S. cerevisiae* var. *boulardii* after the simulated gastric and intestinal fluid testing reported in this study was higher than that reported by [46]; they reported an overall cell survival rate for *L. plantarum* CM53 of 69.09% after sequential exposure to SGF (3 h) followed by SIF (4 h). The probiotics *Wickerhamomyces anomalus* VIT-ASN01 and *S. cerevisiae* VIT-ASN03 displayed 52 ± 0.8 and 59 ± 0.9% survival rates, respectively, as well as the capability of surviving and resisting in gastric fluid juice [18] However, the probiotic yeasts *Saccharomycopsis fibuligera* VIT-MN04 [47] and *Lipomyces starkeyi* VIT-MN03 [48] displayed an 82% survival rate after in vitro digestion and a 90% survival rate after gastrointestinal testing, respectively. An evaluation of the survival capability and viability of *S. cerevisiae* var. *boulardii* after being subjected to simulated gastric and intestinal conditions in vitro is an essential preliminary step before clinical trials. The characterisation of the probiotic paves the path for conducting an investigation and validation using animal models and human trials. It also determines the applicability of the probiotic in functional foods, in supplements, and for biotherapeutic purposes [11].

3.5. Techno-Economic Assessment and Process Simulations at Manufacturing Scale

Using the flowsheet that was created for the 30L development, a simulation of a manufacturing scale (1000 L or 1 m^3) process was designed. The techno-economic evaluation was carried out for both the upstream and downstream processes (Figure 4). The results obtained demonstrated that 4976 kg/batch of probiotic would be obtained once 113 batches were produced as the final product per annum. The cost of manufacturing using the tailored production recipe was lower than that of the imported probiotics used in the country. The results indicated that SuperPro Designer® could be used as a simulation tool in estimating costs and predicting the process feasibility during the production of different bio-based products. By using this tool, the technological feasibility, economic viability, and environmental impact of the process can be fully assessed prior to the fabrication of the production facility [49].

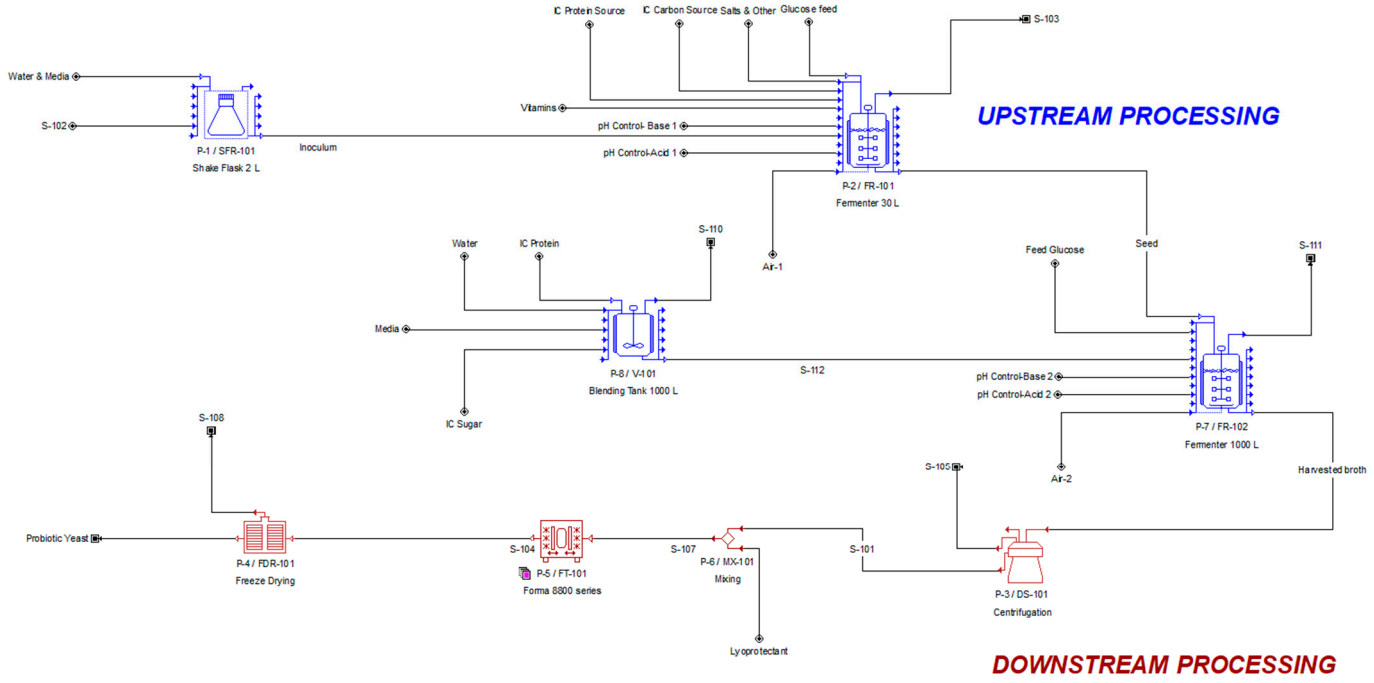


Figure 4. Large scale production of the human probiotic, *S. cerevisiae* var. *boulardii*, proposed for the manufacturing of probiotics in South Africa.

4. Conclusions

This study detailed the production of a human probiotic, *S. cerevisiae* var. *boulardii* conducted in Pretoria, South Africa, presenting the highest known report of the biomass production of this known probiotic using stirred tank bioreactors. The upstream and downstream process unit operations were well integrated to achieve a functionally active freeze-dried product, which was tested in simulated gastrointestinal conditions. The probiotic strain survived and maintained functionality during the production and downstream processing and, importantly, the strain survived the harsh conditions of the human gastrointestinal tract. Additional R&D activities were focussed on compiling simulated manufacturing predictive models using SuperPro Designer software. Once the model was configured, the predicted process performance was interrogated, and the proficiency of the process will be validated by demonstrating the production technology at the manufacturing scale. Upon completion, this will further showcase the country's ability to manufacture functional probiotics for global use, at a cost lower than that of imported goods.

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