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Preparation of freeze dried and vacuum dried yeast starter cultures: evaluation of relevant viability detection analyses

Dried yeast starter cultures have gained considerable importance and popularity in food and beverage industry for the production of different goods. The viability of the preparations used is of utmost importance as this parameter largely influences the manufacturing process and the quality of the later product. However, there is a plethora of possible methods to be applied for the evaluation and making the right choice is often subjective. Therefore, the progress of viability of differently dried yeast preparations was evaluated by different techniques, i. e. colony forming units in relation to total cell concentration, flow cytometry, Oculyze system and NucleoCounter. The processes of freeze drying and vacuum drying were carried out for the production of dry yeast and the obtained preparations were stored at refrigerated and challenging conditions in order to achieve a large range of survival rates. It was found that Oculyze and NucleoCounter measurements clustered at rather high viability levels over the full investigated time, whereas flow cytometry and classical microbiology gave much lower values for the respective samples. The detailed track of the processing course showed that this gap rose with extended processing and storage. The differences can partly be explained by the diverse mechanisms to which the different methods refer. Yet, it can generally be concluded that classical microbiology and flow cytometry were better suited to show a broad range of viabilities. The study data helps users of viability detection methods to make an evidence based decision that represents a good compromise between cost-benefit considerations and the needed level of information.

Descriptors: yeast viability, freeze drying, vacuum drying, viability measurement, yeast preparation, performance test

1 Introduction

Dried active yeast preparations, particularly of the genus *Saccharomyces*, are applied in the bakery-, wine- and brewing industry with increasing popularity [1, 2]. Due to industry goals like prolonged shelf life and the reduction of contamination risks as well as lowering transportation and storage costs [3], the drying of yeast can be an effective preservation method to store and transport yeast at environmental conditions. Different methods to dry microorganisms like vacuum drying, fluidised bed drying, spray drying, and freeze drying are established [4], whereby the latter is one of the most commonly and successfully applied methods for preservation of yeast, fungi and bacteria [5, 6].

Although dried yeast shows a longer shelf life than fresh yeast, yeast viability will be influenced during the freezing and drying process as well as the rehydration [3, 6, 7] and thereby, raise challenges for the production. The use of protective agents can reduce negative effects during freezing and drying by lessening the damaging phenomena accompanied by e.g. phase transition, ice crystal formation and water removal [5]. As mentioned, the application of dry yeast starters gains increased interest for the brewing industry whereby this is also driven by the increased number of small breweries that cannot provide the facilities for yeast treatment and propagation. In practice, it is relevant to avoid both under- and overpitching during the application of dried yeast preparations, as this crucially impairs the fermentation performance and is of economical relevance. Thus, reliable and rapid methods to assess the vitality and viability of yeast are necessary [8]. These can also serve as quality control for producers as well as consumers and thus be applied as a valuable tool during process-oriented quality management. There are several analytical methods for the assessment of microorganisms' viability, whereby the plate count method is the most widely used one [9–11]. This simple and low-cost but time-consuming assay distinguishes live and dead cells based on their ability to divide and grow at given conditions.

Another possibility is the usage of dyes to stain different cell characteristics. Simple staining methods such as methylene blue or methylene violet staining can manually be performed for the distinction of live and dead cells by means of a microscope and a hemocytometer (e.g. Thoma chamber). The principle of both staining methods is the penetration of the respective dye into the cell plasma independent of the cell's physiological status and further dye modification to a colourless component. They are commonly

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Table 1 Definition of yeast viability derived from the analytical method and targeted mechanisms

Method	Mechanism	Derived definition of cell status	Outcome
Plate count	Diluted cells grow under given conditions to visually detectable colonies.	Viable: cells that are able to replicate and form a colony under certain growth conditions.	cfu/mL or g
Coulter Counter	Diluted particles are detected via impedance measurement and assigned to a cell population based on size and volume distribution.	Total cell concentration: Total particle count assigned to a cell population.	cells/mL or g
Combination of plate count and Coulter Counter allows the calculation of the viability			cfu/cells
Nucleo Counter	The dye propidium iodide penetrates only cell membranes with a defect integrity and intercalates in the DNA resulting in fluorescence.	Dead cell concentration as PI-positive. Total cell concentration after lysis of all cells and staining with PI. Viable: Calculated as the ratio of total and dead cell concentration.	cells/mL or viable cells/ total cells
Oculyze system	The dye methylene violet penetrates cell membranes and is autooxidised in the cytoplasm of living cells to the colourless form.	Viable: Cells that have an intracellular environment suited for dye oxidation.	cells/mL or viable cells/ total cells
Flow cytometry	Metabolically active cells transform the dye FDA to fluorescein resulting in a fluorescence signal at 535 nm. PI penetrates only cell membranes with a defect integrity and intercalates in the DNA resulting in fluorescence at 670 nm. Non-fluorescent signals give information about size and granularity.	Viable: Cells with an esterase activity and an intact cell membrane integrity (FDA^+, PI^-). „Damaged/stressed“ cells with an esterase activity and a defective cell membrane integrity (FDA^+, PI^+). Dead: Cells without esterase activity and lost membrane integrity (FDA^-, PI^+). Non-stained cells: Detected cells without any fluorescence signal.	events/ total events

* In general, viability is defined as “living” cells per total cells

cfu: Colony forming units; PI: Propidium iodide; FDA: Fluorescein diacetate

used in breweries for rapid viability estimation [12].

Further, automated stain-based viability and vitality detection can be accomplished via flow cytometry (see also Table 1). Two yeast cell characteristics are commonly used for the assay. On the one hand, the measurement of membrane integrity verifiable with e.g. propidium iodide (PI), which intercalates in cell’s DNA in case of low membrane integrity caused by cell stress, is used. On the other hand, the metabolic activity of cells by e.g. FDA staining is measured, whereby this is proportional to formed fluorescence intensity [11]. PI has the advantage that it can be applied in many co-staining protocols [10]. However, diverse dyes with different staining mechanisms are available nowadays that can be used in multiple staining protocols and allow deepened analyses of physiological changes in yeast over time and the identification of subpopulations with relevant characteristics [9]. The use of flow cytometry is much costlier and more complex in application than plate counting or manual staining and counting and moreover, access to a flow cytometry device is necessary [9].

The aim of the conducted study was the evaluation of different methods for the assessment of viability of a yeast population along the sequential production steps of dry preparations. Thereby, the improvement of process conditions was subordinated to the general characterization of the preparations and the illustration of differences within the analyses. The outcome may help to evaluate the information attainable by the corresponding analytical tools and support operators for an adequate assessment of own results.

2 Material and methods

2.1 Culture conditions

The bottom fermenting strain *Saccharomyces pastorianus* Rh from the VLB strain collection was used for the studies. Three inoculation loops of fresh yeast from a wort agar slant were transferred in 100 mL 12 °P Pilsner wort and grown under shaking conditions at 26 °C for 48 h. This pre-culture was transferred in 7 L fresh wort and incubated under stirred conditions at 26 °C for 48 h. This culture was sampled and then stored at 4 °C for the reduction of cell activity and culture sedimentation. After 3 days of storage, 90 % (v/v) supernatant was decanted and the remaining yeast slurry was used for further investigations. The culture was checked for contaminants via microscoping as well as by coulter counter measurements.

2.2 Preparation of freeze-dried samples

A part of concentrated yeast slurry was mixed in equal volume with the lyo- and cryoprotective solution LyoF, containing (w/w) 1 % glycerol, 5 % maltodextrin (Glucidex 12®, Roquette Frères S.A) and 5 % lactose monohydrate. The mixture was distributed in 1 mL aliquots in 5 mL glass vials and placed on the shelves of the freeze dryer (Sublimator 15, Zirbus Technology GmbH) and freeze dried with an automated profile consisting of a freezing rate of 0.7 °C/min with a minimum temperature of –30 °C. Primary drying was performed at a maximum vacuum of 0.1 mbar, lowered to 0.05 mbar during secondary drying. Additionally, pure concentrated yeast culture was aliquoted and treated as described above without the addition of LyoF. The dried samples were immediately closed with gas-tight caps and stored in glass vials as described below.

2.3 Preparation of vacuum dried samples

Pure yeast slurry was transferred under aseptic conditions on sterile filter papers. These filter papers were put on pug plates

and dried for 4 h at 22 (± 2) °C (room temperature) and a minimal pressure below 0.1 mbar in a vacuum drier (Vacutherm VT6130, Heraeus Deutschland GmbH & Co. KG). Dried filter papers were cut in strips of 2 cm x 10 cm size and placed in closed aluminum bags for storage.

2.4 Determination of low molecular sugars and ethanol

Sugar and ethanol analysis was conducted by HPLC (Knauer Wissenschaftliche Geräte GmbH) applying isocratic elution of 10 µL sample on a Nucleogel® Ion 300 OA column (Macherey-Nagel GmbH & Co. KG) with 5.0 mmol/l H_2SO_4 at a flow rate of 0.4 mL/min and 40 °C column temperature. Detection was performed in a refractive index detector.

2.5 Determination of viability

Determination of viability during yeast preparation and storage was realised by different analytical methods (see Table 1), which differentiate in staining and non-staining principle. Therefore, freeze dried samples were rehydrated in 1 mL sterile 0.9 % sodium chloride solution and filter paper strips were rehydrated in 10 mL sterile 0.9 % sodium chloride solution under aseptic conditions and mixed before the respective analysis.

2.5.1 Plate count method

100 µL of appropriately diluted samples were plated on yeast extract dextrose (YED) agar and incubated at 26 °C for 3 to 5 days before counting colony forming units (cfu). Next to the concentration of cfu per mL, amount per total cell count (cc) measured by coulter counter yielded yeast viability.

2.5.2 Flow cytometry

Flow cytometry (CyFlow® Cube 8, Sysmex Inc.) was conducted by Yeast Control™ - Viability Kit (Sysmex Inc.). Measuring included first incubation with 10 µL fluorescein diacetate (FDA) before samples were stained with 10 µL PI. Direct measuring applying a cell flow of 300 – 3000 events/sec followed. Cells with impaired membrane integrity were stained red via PI and classified as dead cells. Metabolically active cells, classified as cells with existing esterase activity, were stained green caused by transformation of fluorescein diacetate to fluorescein and were determined as viable yeasts. Cells exhibiting both stains still showed metabolic activity but lost their membrane integrity, so that these were counted as damaged cells.

2.5.3 NucleoCounter

NucleoCounter (NucleoCounter® YC-100™, ChemoMetec A/S) measurement was realised by PI pre-filled cassettes (NucleoCassette™). Samples were measured untreated as well as after lysis with Reagent Y100 as recommended by the supplier, whereby only PI stained cells are detected. Thus first dead cells in the sample were detected and further total dead cells after lysis were determined and are representative for the total cell concentration of the sample. Viable cells were determined by subtraction of counted dead cells from total cell concentration, viability was calculated by dividing viable cells by total cells.

2.5.4 Oculyze system

The Oculyze system (Oculyze GmbH) automatically detects viable cells via an image recognition algorithm after methylene violet staining. 100 µL of appropriately diluted samples were mixed with 100 µL 0.01 % alkaline methylene violet solution. After correct setting of the microscope by the operator, five pictures per sample were taken and sent for automated evaluation of total cell concentration as well as live-dead-ratios.

2.6 Determination of total cell concentration by coulter counter

For the analysis of total cell concentration, impedance measurement (Multisizer™ 3, Beckman Coulter GmbH) was used. 10 µL of the original sample were diluted in 10 mL Isoton, and 50 µL thereof were analysed using a 30 µL capillary. The pulse data were converted to size features using the Multisizer™ 3 software version 3.53. Particles of a size of 4 – 10 µm were considered as yeast cells and the concentration of those per mL was calculated.

2.7 Storage conditions and experimental design

Freeze dried (closed vials) and vacuum dried (aluminum bags) samples were stored at 26 °C and 4 °C in a desiccator for 3 months, respectively. Viability and total cell concentration were determined in biological triplicates after 7, 25 and 84 d (26 °C) and 12, 26, 85 d (4 °C), respectively. Samples stored at 4 °C were additionally used after 12 and 85 d for performance tests.

2.8 Performance tests

Rehydrated refrigerated samples were inoculated to a concentration of $2 \cdot 10^6$ cells/mL in 100 mL fresh wort (11.5 °P) and incubated under stirred conditions at 26 °C and 300 rpm. Fermentation studies were done in biological triplicates. The culture was checked for contaminants via microscoping as well as by coulter counter measurements. For offline analysis, 5 mL sample were used and analysed on total cell count via coulter counter, pH-value and residual sugar and ethanol concentration via HPLC.

2.9 Statistical evaluation

All experiments were performed in biological triplicate and data is presented as arithmetic mean ± standard deviation. A two-way analysis of variance (ANOVA) combined with a Tukey`s post-hoc test was performed to demonstrate differences in viability measured via different methods between the production steps of yeast preparations within each viability determination method. Assuming that data are normal distributed, statistical evaluation was performed. Significant differences are defined by a p-value < 0.05.

3 Results and discussion

3.1 Production of dried yeast preparations

The procedure of preparing yeast formulations via two different dry-

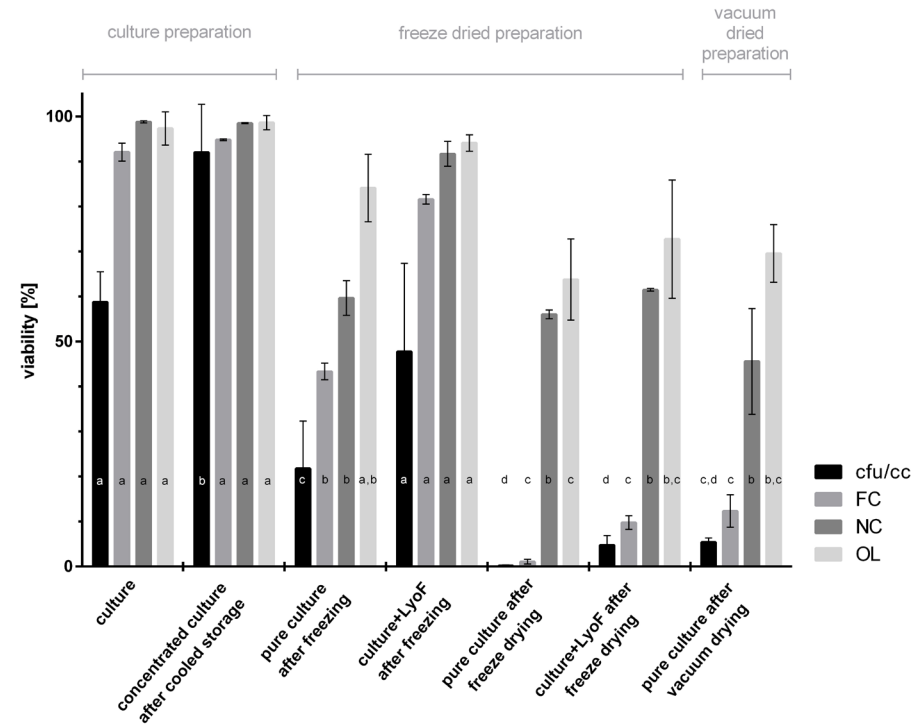

Fig. 1 **Viability of yeast preparations before and after applying different drying approaches. The viability of each sample was determined via colony forming units per cell count (cfu/cc), flow cytometry (FC), NucleoCounter (NC) and Oculyze (OL), respectively. Data is represented as mean value ± SD from biological triplicates. Differences in the viability during the processing steps for each of the applied methods were assessed by two-way ANOVA with Tukey's multiple comparisons test with α-level = 0.05 (different letters indicate significant effects on mean value)**

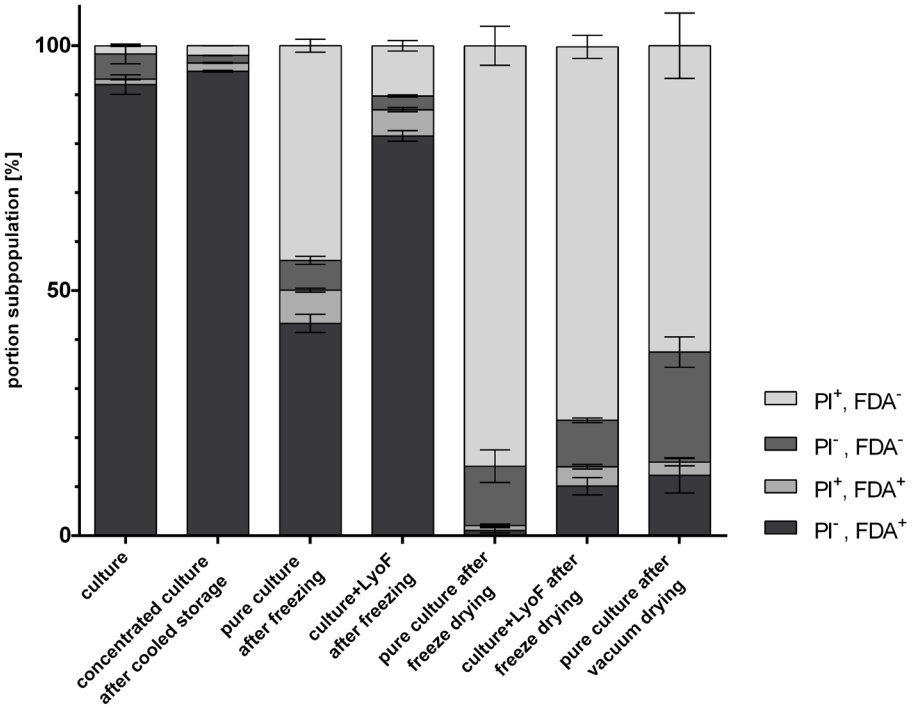

Fig. 2 **Portion of the respective subpopulation of yeast preparations before and after applying different drying approaches. PI^+, FDA^- represents dead cells. PI^-, FDA^- represents unstained cells. PI^+, FDA^+ are metabolically active cells with defective cell membranes defined as damaged cells. PI^-, FDA^+ cells stand for viable cells. Data is represented as mean value ± SD from biological triplicates**

ing strategies was evaluated by comparing the viabilities during and after processing. Figure 1 illustrates the results of the measured viabilities applying different techniques (see Material and methods section).

The applied yeast culture showed a high viability, i. e. 92.1 % ± 1.6 % by flow cytometry to 98.8 % ± 0.2 % by NucleoCounter after propagation as well as after concentrating which could be seen with all the applied methods. Only the cfu/cc directly from the culture was surprisingly lower but this effect was not observed after cooled storage and concentrating anymore.

It can firstly be seen that the freezing of the samples influenced their viability, whereby the usage of the cryo- and lyoprotective matrix LyoF was generally beneficial resulting in significantly ($p < 0.0001$, except for Oculyze) higher survival rates (27 % on average) compared to freezing without protection matrix. Secondly, the drying process impaired the viability of the yeast samples, whereby also the application of LyoF showed better outcomes (7 % on average compared to pure culture). It is generally known that protecting agents can have positive effects on yeast viability during preparation of freeze dried yeast as dehydration stress by osmotic pressure can be reduced [5, 13, 14]. Freeze drying of yeast with protection matrix and vacuum drying of pure yeast showed almost the same viabilities for the respective determination methods. The sublimation of ice approached within the freeze drying process is considered a very gentle method to dry goods [15]. However, from the results it can be assumed that gentle drying can only compensate the negative consequences of freezing the samples. In contrast, when applying vacuum drying at room temperature, the water only evaporates from the samples avoiding freeze-stress and thus superseding protection matrices. This is then justified by the same viability levels of those two kinds of samples. *Cerrutti* et al. [16] described in investigations of commercial baker's yeast a lower loss in viability with vacuum dried yeast samples at 40 °C in comparison to freeze dried samples.

In comparison to the concentrated fresh culture, a reduction of viability by approximately 20 % referring to Oculyze up to approximately 90 % referring to colony

forming units per overall cell count was seen for the freeze dried protected yeast as well as the vacuum dried yeast after drying. Accordingly, different analytical methods showed large variations in viability values for the same samples. In general, there were two groups of viability levels observed: rather high levels measured by the NucleoCounter and Oculyze and quite low levels determined by flow cytometry and classical microbiology. These trends presumably originate from the underlying measurement principles and the resulting measuring range. The ability to form a colony out of a single cell is the most conservative way of defining viability taking the highest cell activity as a basis. This is especially true when the total cell concentration measured via particle analyser is consulted for the calculation of the cfu/cc ratio. Via the used staining-based flow cytometry assay, the metabolic activity as well as the membrane integrity were considered simultaneously and thus not only discriminating dead vs. alive but also damaged cells that are still metabolically active but may not be able to divide (further consideration in Fig. 2). The latter may also be able to repair their membranes and then further contribute to cell forming units [17]. This may also affect the stain-based viability measurement via NucleoCounter, which only considers the membrane integrity. Despite the staining mechanisms being different (see also Table 1), the Oculyze system and the NucleoCounter gave similar results. This is in good accordance to former studies concerning fresh yeast cultures, where the Oculyze system was applied with methylene blue and compared to manual counting by hemocytometer and NucleoCounter [18]. Those studies showed good correlations of the three methods, especially at high viabilities.

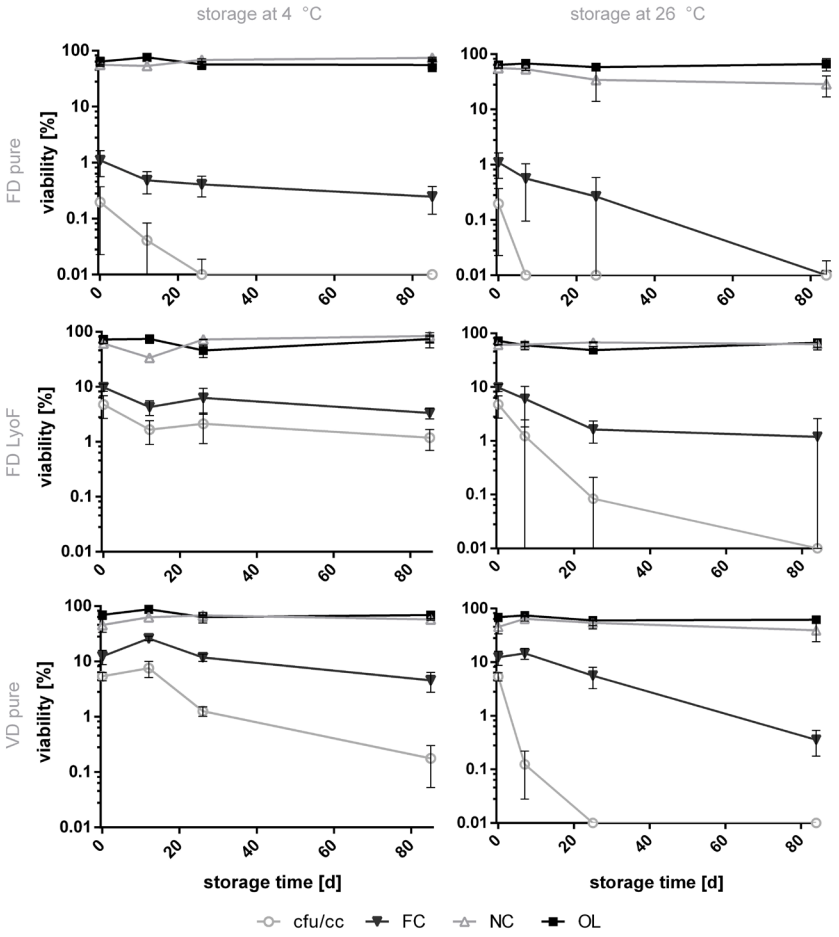

Fig. 3 **Progress of viability of vacuum dried (VD) and freeze dried (FD) yeast preparations detected via diverse viability detection methods during storage. The viability of each sample was determined via colony forming units per cell count (cfu/cc), flow cytometry (FC), NucleoCounter (NC) and Oculyze (OL), respectively. Data is represented as mean value ± SD from biological triplicates**

Figure 2 shows the information gained by the flow cytometry analyses in more detail, whereby four subpopulations per yeast sample are classified. Cells defined as viable are conservatively described as those showing metabolic activity (FDA^+) and intact cell membranes (PI^-). This subpopulation can also be detected via cfu/cc whereas dead cells (PI^+) should be in accordance to NucleoCounter measurements. The Oculyze system detects viable colourless cells without considerations of membrane damages. As the metabolic activity is not explicitly distinguished by NucleoCounter and Oculyze in terms of direct detection of enzymatic activity, it can only be considered in flow cytometric measurements (FDA^+, PI^+). So it can be seen, that cells after freezing independent of the influence of the protection matrix show around 6 % metabolically active cells with damaged cell membranes.

Although the amount of viable cells in pure cultures after vacuum drying is comparable to the viable cell concentration of freeze dried LyoF samples, the proportion of dead cells is considerably lower. Nevertheless, it is noticeable that the vacuum dried samples have a comparable amount of damaged but metabolically active cells. Thus, this subpopulation could have potential to be targeted in further developments. The amount of unstained cells in the vacuum dried samples is more than twice as high compared to freeze dried samples. The impact and potential of this subpopulation in performance tests might be interesting, so that in further investigations these cells could be sorted and analysed separately.

The subsequent effects on the final results are further discussed concerning the subsequent storage studies presented in the following section.

3.2 Storage of dried yeast preparations

The yeast preparations described in section 3.1 were subsequently stored at 4 °C and 26 °C for three months. For the evaluation of their stability, samples were periodically taken and analysed via the same analytical methods as described above. Figure 3 shows the progress of yeast viability for the different formulations over the storage time. It is obvious that the samples stored at 26 °C lost their viability more rapidly than those stored at 4 °C independent of the applied analytical method. Cooled storage is generally fa-

vorable as then biochemical reactions inside the cells are naturally slowed down [19]. The storage at 26 °C aimed for two objectives: mimicking unfavorable conditions to show negative effects on yeast when e.g. shipping without adequate temperature control or improper handling in general as well as serving as an accelerated shelf life test [20]. Thereby, viability behavior at inconvenient shipping or storage conditions is of high industrial relevance as each percent of saved viability means saved money as well. As not all commercially available yeast preparations may constantly meet their given guarantees [21], suppliers seek to improve that state. It can generally be concluded from the data that the effects of cooled storage on viability after three months could already be seen after one to three weeks of storage at room temperature, which illustrates the applicability of accelerated shelf life tests for quality assurance. In the following, as the tendencies between the different sample types were the same independent from storage temperature, the samples stored at 4 °C, which is industrial practice, will be discussed in more detail.

The general trends between the different sample types that were observed directly after freeze drying also manifest during storage. The LyoF-protected yeast samples showed smaller changes in viability during storage time than pure freeze dried and vacuum dried samples. Yet, the vacuum dried preparations showed only slightly lower viabilities. Pure freeze dried yeast presented the lowest survival rates on average, which is most probably because they experienced the highest damage by the diverse harsh phenomena during freezing and drying like ice crystal formation, phase transition, gradient formation and water removal without counteracting with protectants [5, 22]. The fluctuations in viability after one week of storage especially for protected freeze dried as well as vacuum dried yeast samples at 4 °C are noteworthy. Thereby, for the protected freeze dried yeast samples the viability after one week is reduced and restored two weeks later. This may be due to a certain regeneration of cells, e.g. by internal repair mechanisms, which take place after stressful conditions such as changing the environmental conditions [17]. This may also explain why freeze dried and vacuum dried preparations behave slightly different: the vacuum dried cells were processed at room temperature and thus experienced an environmental change when going to storage at 4 °C whereas freeze dried preparations underwent very low temperatures and were then set to comparably higher ones.

Again, large differences between NucleoCounter and Oculyze on the one hand and plate counting and flow cytometry analysis on the other hand occurred. Overall, viability determination by Oculyze and NucleoCounter only showed small changes in viability of all samples, even after prolonged storage. Oculyze, which means automated image recognition of methylene violet stained cells as well as colourless cells, is mainly influenced by the image recognition algorithm itself. Generally, simple manual staining methods comprise the evaluation of a comparably low amount of cells, so that the analysis of yeast viability is more subjective and might be unrepresentative in contrast automated measurement as with e.g. flow cytometry [23]. Furthermore, cells, which do not show any structural modifications as e.g. disrupted cell membrane yet, but other lethal damages, are not detected via this method [24]. Therefore, viability detection based on staining with methylene blue as well as methylene violet can lead to overestimated viability rates [5]. When applying Oculyze, the cell counting and distinction of dead from viable cells is automated, which substantially reduces manual errors by the experimenter. However, the crucial steps are the consistence in the staining itself, which is mainly influenced by the staining solution and incubation, and a sufficiently high level of focusing of the images by the operator. Together, those two factors determine the contrast of the cells and thus the final results. While the overall cell concentration determined via Oculyze was in good accordance to impedance measurement by coulter counter (data not shown), the respective portion of dead cells was underestimated. This might be due to the methylene violet staining. Methylene blue, which has the

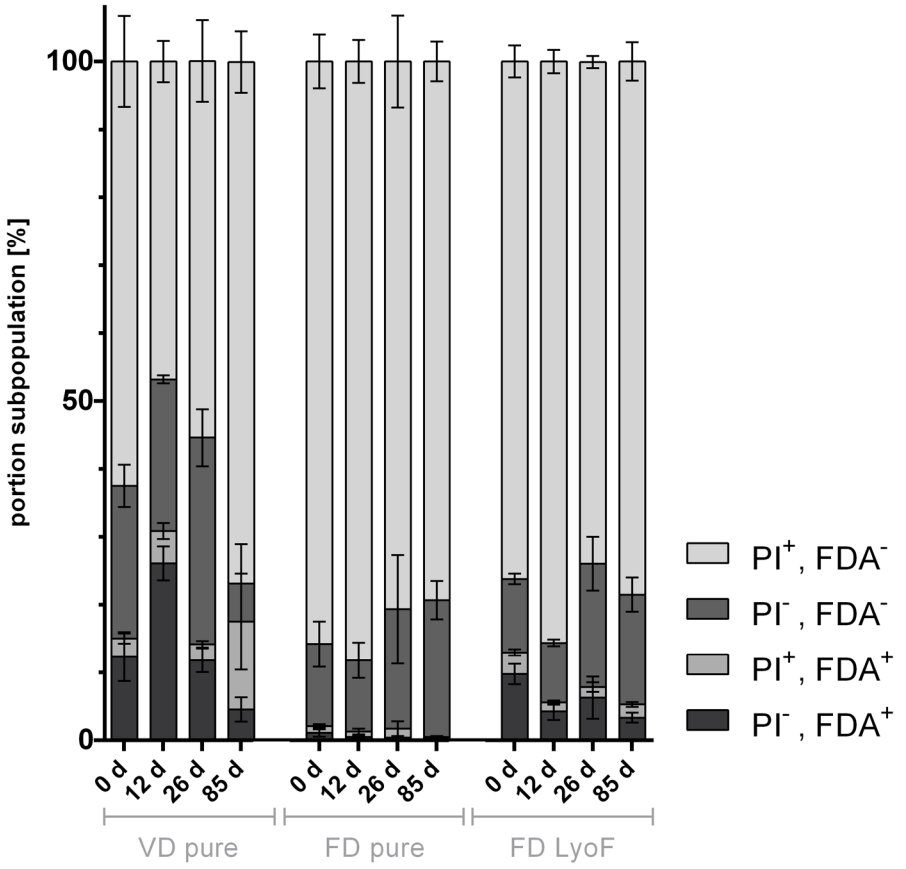

Fig. 4 **Portion of the respective subpopulation of vacuum dried (VD) and freeze dried (FD) yeast preparations detected via flow cytometry during storage at 4 °C. PI^+, FDA^- staining represents dead cells. PI-, FDA- represents unstained cells. PI^+, FDA^+ are metabolically active cells with defective cell membranes defined as damaged cells. PI^-, FDA^+ cells stand for viable cells. Data is represented as mean value ± SD from biological triplicates**

same mode of action [12], was recommended by the Institute of Brewing as an alternative rapid procedure for highly viable yeast samples [25]. However, it is known, that this method cannot be correlated to culture based methods when having amounts of less than 40 % death cells as it takes into account also cells that will not reproduce but are metabolically active [26]. Nevertheless, as especially methylene violet was proven to better represent viability compared to methylene blue [12], this might not be the only explanation. It is quite likely that the Oculyze procedure with the above-described obstacles contributed to the comparably high viabilities.

Plate counting represents the most conservative way of assessing microbial viability and thus still serves as the gold standard for producers as well as users of starter cultures [9]. Viability is here defined as the ability of a single cell to form a colony. Yet, one has to keep in mind that also cell clumps, which is especially true e.g. for pseudomycelium-forming top-fermenting yeast, may form only one single colony. Further, metabolically active cells that show up with impaired cell membranes may not form a colony or at least not under the given conditions and are then considered as dead [11], but contribute to the final product appearance. Thus, the assay is not suited to detect those kind of cells, leading to an underrating of living cells [9]. As a consequence, the viability via plate counting assay is always underestimated to a certain degree which is also substantiated by the presented data in this paper. Plate count results showed the lowest viability ratios independent from the investigated type of preparation.

Flow cytometry showed the most comparable results to plate counting. The technique itself allows for rapid assessment of a large and thus representative amount of cells in a short amount of time [23]. By that, a more rapid single-cell analysis with higher informative value based on objective identification of subpopulations via automated cell counting of several thousand cells per second is achieved [9, 11, 23]. The applied measurement kit makes use of two different dyes, i. e. FDA for metabolic activity as well as PI for membrane integrity. Consequently, more information can be obtained from one single measurement (Fig. 4).

Figure 4 shows the progress of the distribution of subpopulations of different dried yeast preparations stored at 4 °C. Former studies with *Saccharomyces* species showed that cells with enzyme activity and intact cell membranes (illustrated as well in Fig. 3) can be considered as colony forming units as well [27]. However, the cfu/cc was constantly lower than the FDA^+, PI^- subpopulation, justified, as explained above. As already described, metabolically active but damaged cells are interesting in context of their impact in fermentations. When considering this subpopulation (FDA^+, PI^+), which could be able to influence the fermentation performance of dried preparations, a minimally higher amount of cells can be observed at all samples. As can be seen, especially the vacuum dried samples stored for 85 days showed around 10 % of all cells in this subpopulation. Against expectations, these samples showed a lower performance in following performances tests after 85 days of storage compared to freeze dried LyoF samples (Fig. 5), which showed a lower part of viable and damaged cells in sum. It is possible, that the rehydration step was insufficient, so that the damaged cells were not able to repair under these conditions. Another interesting aspect is the number of cells that are not stained with either of those two dyes. While with vacuum dried samples the amount of unstained cells stayed constant, except for samples stored the longest, their amount increased with pure freeze dried samples as well as protected freeze dried samples during storage progress. This can be caused by cell stress during processing leading to absence of metabolic activity or low intracellular pH while keeping intact cell membranes. The fluorescence of fluorescein is pH dependent and thus the intracellular pH of the yeast cell itself affects the fluorescence intensity [28]. This subpopulation could later on potentially contribute to active yeast and their reactivation would be of high industrial interest but is left open to further research.

For the assessment of membrane integrity via PI one has to take into account that also stressed cells are permeable to PI, but may later on restore their integrity [17]. This is especially critical when rehydrating dried yeast as shown by *Davey* et al. [17] for baker's yeast. It took about 60 min for the yeast to fully recover. Such swelling periods have to be kept in mind when analysing dry yeast preparations via PI to avoid false to high amounts of dead cells. The NucleoCounter method finally also relies on PI staining. Consequently, the viable cells were only measured indirectly. The calculated viability values were surprisingly high. It was expected, that the proportions of PI-stained cells measured with the NucleoCounter would be in a comparable range to those gained from flow cytometry. However, that was not the case. Within the NucleoCounter assay, a cell lysis step is conducted to cover the overall cell concentration. As those concentrations were comparable to the other applied methods (data not shown) it is more likely that the record of dead cells is false too low.

A closing general aspect is the number of gathered events for each of the methods. For colony forming units, NucleoCounter and Oculyze, comparably low amounts of events in a range of several hundred are detected. Whereas the Oculyze system identifies cells based on their shape and subsequently distinguishes their colour, the NucleoCounter is only able to detect fluorescence signals of dead cells and thus results may also be based on a rather low number of events. Consequently, a higher statistical error is included in those measurement techniques spreading the standard deviations. In contrast, via flow cytometry a high amount of cells can be detected in a short time reducing the susceptibility to high statistical errors.

Concluding, the choice of the applied analytical method for determination of viability depends on the envisaged sensitivity and level of information that is to be gathered from it. It is further necessary to assure, that the method used is able to cover the expected measuring range. Consequently, operators who need a deep insight into their cell characteristics, such as starter culture producers, should depend on methods that are able to provide such information. Also if certain analyses are mandatory due to regulatory standards, methods like e.g. flow cytometry can be a time saving add on for product quality approval. On the other hand, manufacturers can apply easier-to-use methods as e.g. Oculyze or NucleoCounter in standardized industrial processes that are carried out with a certain degree of reproducibility or when high viability levels are to be expected.

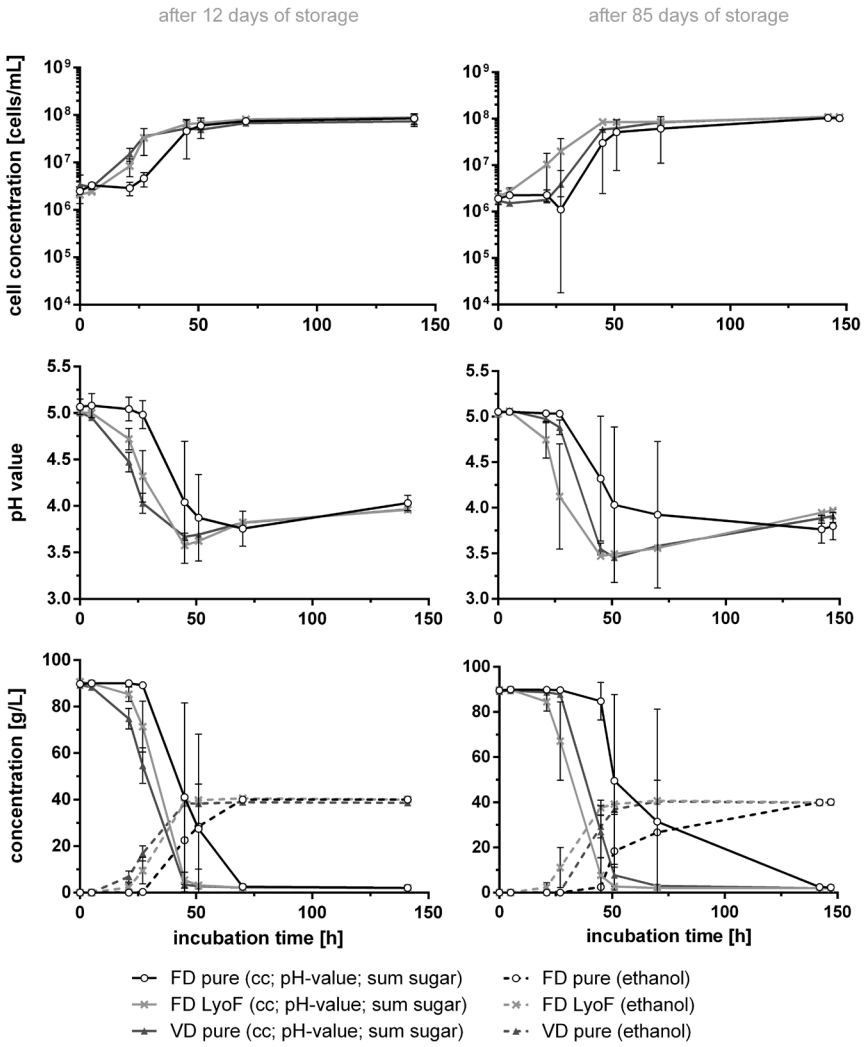

Fig. 5 Performance of differently produced dry yeast preparations by vacuum drying (VD) and freeze drying (FD) after short- and long-term storage at 4 °C. Data is represented as mean value ± SD from biological triplicates

3.3 Performance of dried yeast preparations

The differently produced yeast preparations were also practically applied as starter cultures for fermentation studies. Thereby, the effect of the method of preparation as well as the influence of the storage time was investigated. The progress of cell count, changes in pH-value as well as sugar uptake and ethanol production were examined and visualised in figure 5.

After short-term storage of 12 days, highest performance was achieved by vacuum dried yeast close to freeze-dried yeast with protection matrix, as there the fermentation progress was comparably fast. Pure freeze dried yeast exhibited lower performance in growth, acidification, sugar uptake and ethanol production rate. Thereby also the highest values of standard deviation (n = 3), were reached. After 72 h of incubation all samples achieved the same cell concentration of $8.2 \cdot 10^7$ cells/mL on average and equal ethanol concentrations of 40 g/L ± 0.6 g/L with a residual sugar concentration of 2 g/L.

Dried yeast preparations stored for three months showed slight differences in performance in comparison to short-term storage. Until the end of incubation time, little higher values of cell count and higher pH decrease could be observed by all dried yeast preparations compared to short-term storage performances whereby all samples achieved equal levels of the respective parameters.

The highest performance was achieved by freeze dried yeast with protection matrix exhibiting faster growth, acid production, sugar uptake and ethanol production compared to pure freeze dried and vacuum dried yeast preparations as well as compared to performance of same sample after short-term storage (Fig. 5). Vacuum dried yeast samples showed a lower performance compared to short-term storage. The slowest performance characterised by high standard deviations, which were caused by one sample, which did not show any yeast growth within an incubation time of > 72 h, occurred with pure freeze dried yeast preparations. However, by the end of incubation time, equal results were obtained for the preparations.

The apparent general trends like positive effects of the protective matrix LyoF as well as the promoted viability loss with extended storage time are according to expectations. Nevertheless, with the here investigated diverse analyses, it is possible to connect the multiple physiological information and the corresponding principle of viability calculation with the growth performance of the preparations used as direct starters.

It can be seen that the order of the fermentation performance for both investigated storage times is readable from the order of the viabilities determined via cfu/cc and FC, respectively (Fig. 3 and 5). This association is not clearly feasible by NC and OC. For instance, the two freeze dried sample types showed after 85 d of 4 °C-storage distinct differences in all viability measurements via FC and cfu/cc whereby the latter analysis showed again the most conservative results with very low viabilities below 0.001 % for the pure lyophilisates (FD pure) and around 1.2 % for the preparations with LyoF. The positive effect of the protective matrix was also clearly visible in the performance test. In contrast, the detected viabilities measured via OC (FD pure: 75 %, FD LyoF: 84 %) and NC (FD pure: 56 %, FD LyoF: 74 %) gave no clear evidence for the differences in performance (see Fig. 3 upper left and middle left; Fig. 5). Further, next to the relatively low performance of the pure freeze dried sample on average in comparison to pure vacuum dried and LyoF protected freeze dried samples, it also showed a high deviation in the triplicate tests.

Within the study it was intended to investigate a wide span of yeast viabilities in order to illustrate features of the different analyses.

It was shown that the corresponding values for viability can differ over several magnitudes. Thereby it is interesting to see that also preparations with a strongly diminished concentration of cfu/cc can perform in fermentation only with little deficits (e.g. Fig. 3 and Fig. 5, VD pure 85 d 4 °C).

4 Conclusion/Summary

In this study two types of freeze dried and one vacuum dried yeast preparation were produced and the progress of yeast viability at each step of production was evaluated by different analytical methods. Thereby, the motivation was to specifically compare the analytical methods over a wide range of cell viabilities and derive knowledge that is relevant for practice. During the preparation of dried yeast via freeze drying the expected positive effect of a protective matrix was determined. This stability enhancing effect was also detectable during storage of the samples at 4 and 26 °C. However, the investigated viability measurements showed clear differences in the absolute values. This phenomenon increased with proceeding processing and storage time of the yeast. Based on the determined viability values, two methods, NucleoCounter and Oculyze system can be grouped against colony forming units per cell count and the here performed flow cytometric assay. Thereby the two former methods tended to result in higher portions of viable cells whereby the two latter methods gave information over a broader range, thus are more sensitive for samples with high amounts of dead and damaged cells. It is obvious, that the determined differences between the methods are due to different mechanisms of action on one side, and the principle of the final viability calculation on the other side. It is important to understand the kind of information gained from the applied analysis and therefore to know the exact definition of viability as determined by the chosen method. This way, operators can specifically use the information and choose an adequate analytical tool for the corresponding application. Thereby the balance between expressiveness, sensitivity and benefit of each analytical method in context with further application of dried yeast preparations must be found.

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