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# Quantification of oxidative stress in *Saccharomyces pastorianus* propagation: Gene expression analysis using quantitative reverse transcription polymerase chain reaction and flow cytometry

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When confronted with environmental stress, yeast cell reacts, among others, by modifying the expression of specific genes. In this study, gene expression was analyzed *via* RT-qPCR to quantify the oxidative stress of *Saccharomyces pastorianus* during yeast propagation as a reaction to different aeration levels. Target genes were identified, and a reference gene system was developed. Fermentation experiments were conducted in shaking flasks, applying different shaking speeds to generate various aeration efficiencies. The cells were sampled at different propagation stages and, additionally to the expression study, analyzed by flow cytometry after staining with dihydroethidium (DHE) to quantify reactive oxygen species (ROS) inside the cells. The results indicate that high oxygen fermentation conditions led to an increased expression of the catalase-A gene CTA1 during propagation. Furthermore, the determination of cell internal ROS shows increasing oxidative stress over the process in accordance with the RT-qPCR measurements.

## KEYWORDS

aeration, flow cytometry, oxidative stress, yeast propagation, reactive oxygen (ROS), qPCR, *Saccharomyces*

## 1 Introduction

*Saccharomyces pastorianus* is a facultative anaerobic organism that can alternate its metabolism between respiration and fermentation. Since the oxidative pathway yields more ATP, this yeast requires the presence of oxygen in the cultivation medium for optimal growth conditions (Alexander & Jeffries, 1990). During multistage metabolic processes, such as respiration, steroidogenesis, and  $\beta$ -oxidation of fatty acids, various

partially reduced forms of molecular oxygen are formed as by-products (i.e., hydrogen peroxide, hydroxyl radicals, and superoxide anions) (Jamieson, 1995; Jamieson, 1998). These intermediates are called reactive oxygen species (ROS). ROS can react with various intracellular molecules and affect the whole organism by damaging DNA, lipid structures, and proteins, even triggering apoptosis. (Bayir, 2005; Dickinson & Schweizer, 2005; Perrone et al., 2008). Reactive oxidants can cause damage to DNA and nucleoproteins through base and sugar alterations, the introduction of abasic sites and strand breaks, and DNA-protein cross-links (Moradas-Ferreira et al., 1996). One of the main reactions forming ROS is the iron-catalyzed Haber-Weiss and Fenton reaction, with hydrogen peroxide as a key molecule and the highly reactive hydroxyl radical as a product that can directly interact with DNA (Frankenberg et al., 1993; Baynes & Dominiczak, 2019). High concentrations of hydrogen peroxide lead to lipid peroxidation and affect the structural integrity of membranes by increasing membrane fluidity and shortening fatty acyl chains (Steels et al., 1994). Increased membrane fluidity was shown to correlate with higher stress susceptibility (Ishmayana et al., 2017). ROS, and especially hydroxyl radical exposure, result in huge damage due to reactions with the alpha carbon and the amino acid side chains on the protein side. The radical reaction promotes chain reactions or cleavage of the protein backbone (Toledano et al., 2003). Most ROS are generated in the mitochondrial respiratory chain, where 85%–90% of the available oxygen is consumed (Halliwell & Gutteridge, 2005).

Consequently, aerobically growing brewer's yeast is permanently exposed to reactive oxygen species (Lushchak & Gospodaryov, 2005). Given the omnipresent character of ROS, yeasts have developed protection for their cellular components against reactive oxidants (Jamieson, 1998). Under normal conditions, there is a balance between reactive oxidants, antioxidant buffering capacity, and defense mechanisms capable of maintaining homeostasis.

The two parts of the cellular reaction to oxidants are primary defense mechanisms that neutralize ROS, while secondary mechanisms repair oxidation damage. In the case of exceeding ROS levels compared to antioxidants, the cell experiences oxidative stress (Moradas-Ferreira et al., 1996). Next to by-products of oxidative phosphorylation, oxidative stress can directly be caused by the take-up of dissolved oxygen (DO) from the medium. Yeast cells deploy different defense mechanisms depending on ROS dose (Temple et al., 2005). Lower amounts of ROS cause adaptation, leading to more robust cells against further oxidative stress (Jamieson, 1992). Significant exposure induces the expression of antioxidants and delays the cell cycle, while even higher concentrations initiate apoptosis in parts of the culture or lead to necrosis (Lee et al., 1996; Perrone et al., 2008). To constantly maintain a low ROS concentration, yeast relies on enzymatic and nonenzymatic antioxidant defense mechanisms. The radical scavenger

glutathione is a quintessential member of the nonenzymatic class of antioxidants. The redox-active sulphhydryl group of glutathione reacts directly with oxidants and reduces them to less harmful molecules (Moradas-Ferreira et al., 1996). One significant enzymatic way to control the ROS level in the cell utilizes metal-dependent enzymes like catalases. Catalase is an iron-containing enzyme with a haem group in its active site. *S. cerevisiae* contains two catalases: cytosolic CTT1 and peroxisomal catalase CTA1. Catalase reduces hydrogen peroxide to water and oxygen using a dismutation mechanism (Toledano et al., 2003). Adequate aeration and low ROS concentrations must be balanced for good aerobic growth conditions. Thus, the oxygen supply for yeast propagation is crucial due to the versatile function of oxygen in yeast cell metabolism and its essential role in enhancing unsaturated fatty acids and ergosterol biosynthesis, both vital molecules for cell growth (Verbelen et al., 2009). *S. pastorianus*, a hybrid of *S. cerevisiae* and *S. eubayanus*, is one of the industrially most relevant yeasts (Monerawela & Bond, 2018). Despite this, knowledge about the organism is mainly derived from the apparent similarity to *S. cerevisiae*. For industrial brewing applications, yeast is cultivated in propagator vessels in preparation for the following beer fermentation. Cell counts increase 10-fold up to  $100 \cdot 10^6$  cells during this aerobic process. This work aims to evaluate the response of *S. pastorianus* to ROS during brewer's yeast propagation. The expression of catalases as the primary protection mechanism against hydrogen peroxide was monitored using RT-qPCR measurements. Furthermore, the ROS content was estimated using fluorescence dye as a reference method.

## 2 Materials and methods

### 2.1 Fermentation

Fermentations were performed with *S. pastorianus* TUM 34/70 in 500 ml baffled shake flasks at 28°C. The initial filling volume was 40 ml of fresh wort in addition to 10 ml preculture, resulting in an initial cell count of  $2.1 \cdot 10^7 \pm 8.7 \cdot 10^6$  cells. In the brewing industry, “extract” and “free amino nitrogen (FAN)” are terminologies for carbon and nitrogen sources available for the yeast. The wort used for this study had an extract content of  $11.28 \pm 0.11$  °P,  $197.6 \pm 3.69$  mg/L FAN, and a pH of  $5.14 \pm 0.036$ . The hopped wort was provided by an industrial brewery during knockout. Cultures were grown at 100 rpm, 150 rpm, 200 rpm, and 250 rpm shaking speeds for different aeration levels, resulting in  $k_{La}$  values ranging from 51 to  $180 \text{ h}^{-1}$ . Samples were taken after 0 h, 4 h, 24 h, 48 h, and 72 h. Dissolved Oxygen (DO) in shake flasks was measured with a noninvasive optical sensor (SFR Vario, PreSens Precision Sensing GmbH, Regensburg). The  $kLa$  values were calculated using the  $kLa$  calculator from PreSens Precision Sensing GmbH, which is

TABLE 1 The target primer set used for RT-qPCR amplification (Verbelen et al., 2009).

Gene	Direction	Primer sequence (5'-3')	Function
CTA1	Reverse	GGCCATTCCATACCTCTTG	Catalase A
	Forward	TCCGCTATCAGAGATGGAC	

intended for combination with the shaker used (PreSens Precision Sensing GmbH, 2022).

## 2.2 Analysis of relative gene expression

### 2.2.1 RNA extraction and cDNA synthesis

Sampling was conducted according to literature (Dragosits et al., 2011). The Monarch Total RNA Miniprep Kit (New England Biolabs, Ipswich, United States) was used for RNA extraction. RNA purity and integrity were controlled by denaturing agarose gel electrophoresis and capillary electrophoresis (Bioanalyzer; Agilent Technologies). The denaturation of 5  $\mu$ l RNA was carried out using a buffer consisting of 2  $\mu$ l MOPS  $\times$ 10 buffer (200 mM MOPS, 50 mM sodium acetate trihydrate, 10 mM EDTA Quantification), 10  $\mu$ l Dimethylformamid at a temperature of 65°C for 10 min. The electrophoresis was performed at 70 V/cm in  $\times$ 1 MOPS buffer. Additional quality control was evaluated using spectrophotometry (NanoDrop; Thermo Scientific). The visibility of clear 28S and 18S rRNA bands on the gel was used to indicate minor RNA degradation. Additionally, the RNA integrity number (RIN) was evaluated. cDNA synthesis

was conducted according to New England Biolabs (New England Biolabs, Ipswich, United States) first-strand cDNA synthesis protocol (NEB#M0368).

### 2.2.2 Primers

Table 1 shows the primer set for the selected target gene CTA1. According to the literature, possible reference genes were selected from different metabolic pathways, as displayed in Table 2, to minimize possible coregulation [25, 28, 29]. The *Saccharomyces* genome was taken from the *Saccharomyces* genome database due to the hybrid genetic character of *S. pastorianus* (Cherry et al., 2012). Primers were designed using Clone Manager nine software (Sci Ed Software) and synthesized using TIB Molbiol Syntheselabor GmbH (Berlin). Primer efficiencies were determined using a 1:5 cDNA dilution series of five steps (Rasmussen, 2001). A no-template control (NTC) was also measured for each primer pair. All dilutions and NTCs were determined in triplicate. For known pseudogenes, primers were chosen according to the alignment results between genes and pseudogenes. In this direction, the primers were unique to the genes of interest. The secondary structure of the amplicons was analyzed with a Mfold using the criteria  $-3 < \Delta G$ .

### 2.2.3 qPCR and analysis of expression stability

qPCR measurements were conducted in a final volume of 10- $\mu$ l containing 1  $\mu$ l of DNA template, 0.4  $\mu$ l of each respective primer, 5  $\mu$ l of SybrGreen (Biozym, Germany), and 3.2  $\mu$ l of RNase-free water.

Gene expression was measured by real-time RT-qPCR, and the expression stabilities were evaluated by the M and V values based on the geNorm algorithm using the software qBase+ (Biogazelle, Gent, Belgium) (Vandesompele et al., 2002). The

TABLE 2 Primer sets used for RT-qPCR amplification as possible reference genes.

Gene	Direction	Primer sequence (5'-3')	Function
UBC6	Reverse	TTGTTTCAGCGCGTATTCTGTC	Ubiquitin-conjugating enzyme
	Forward	ATGCGGCAAATACAGGTGATG	
TAF10	Reverse	CACCGTCAGAACAACTTTGC	Subunit of TFIID and SAGA complexes
	Forward	TAACAACAGTCAGGCGAGAG	
ALG9	Reverse	CTCTCGGAGGTACAACCTG	Mannosyltransferase
	Forward	CACTAAATCAAGGTGGTGTGAAG	
TFC1	Reverse	TCGTCTGGCTCTTCAGTTATGG	Subunit of RNA polymerase III transcription initiation factor complex
	Forward	TGGATGCAGGTAGTCAAATGTC	
PGK1	Reverse	TTCGTCTAACAAAGCCTTAGTACC	3-phosphoglycerate kinase
	Forward	CTAGAAAGTTGTTTGTGCTACTG	
KRE11	Reverse	CTCTCGGAGGTACAACCTG	Component of transport protein particle complex II
	Forward	ATTCGCCCTTGACACTGG	
PFK1	Reverse	AACCTTAGCGTCAGTGTGTTGG	$\alpha$ -subunit of heterooctameric phosphofructokinase
	Forward	CATGTTCAACAAGGTGGTGTTC	
YRB1	Reverse	CTGCTTACCTTCTGCAATATC	Ran GTPase binding protein
	Forward	CCACATCATTGCTCCAGAATAC	

Cq values from RT-qPCR were imported into qBase Plus software (Biogazelle). Normalized relative quantities were calculated using the modified Pfaffl method with multiple reference genes (Hellemans et al., 2007).

## 2.3 Determination of reactive oxygen species

A Cytotflex flow cytometer (Beckman Coulter GmbH, Krefeld) equipped with an argon-ion laser operating at 488 nm was used in the experiments. The cytometer was fitted with four sensors for front and side scatter detection of green (525 nm, channel 1), yellow (585 nm, channel 2), orange (610 nm, channel 3), and red (780 nm, channel 4) fluorescence. The cells harvested simultaneously for PCR analysis were washed three times in a Ringer solution, and the optical density was set to 0.1. The staining of the cells was conducted with dihydroethidium (DHE), according to Landolfo et al. (Landolfo et al., 2008). The fluorescence was detected at 610 nm, and at least  $2 \cdot 10^5$  cells were analyzed in each experiment. Further data analysis and visualization were performed with MATLAB (The MathWorks, Natick, United States). The typical evaluation of flow cytometry data is the calculation of histograms with an event count over logarithmically scaled fluorescence. One significant disadvantage of this technique is the artificial categorization of continuous data. Here, the choice of intervals has a decisive influence on the representation and possible evaluation based on the histograms. A more elegant method is the use of so-called Kernel density estimates. These enable a continuous estimation of an unknown distribution of data points. Calculating the probability density function enables discretization of the data and a more accurate and independent display of the results by creating continuous data. The estimate calculation was based on Eq. 1 shown below, where  $n$  = sample size,  $h$  = bandwidth, and  $K$  = Kernel function. Usually, the first step would be to choose the best fitting Kernel function between Gauß-, rectangular-, triangular-, and Epanechnikov-Kernel. In the case of flow cytometry, the vast amount of evaluated data points makes the difference in the resulting estimation negligible. In this study, the Epanechnikov-Kernel (Eq. 2) was used. However, the bandwidth choice for the density function distribution calculation makes a significant difference. The larger the bandwidth, the smoother the function, and the poorly represented the actual distribution. Various methods exist for calculating the optimal bandwidth, whereby the method, according to the Eq. 3 by Silverman, is used here (Silverman, 2018). This method is a fast, sufficiently good approximation of the optimal bandwidth and depends on the standard deviation of the data sets and the interquartile range.

$$\hat{f}_h(x) = \frac{1}{nh} \sum_n^{nh} K \left( \frac{x - x_i}{h} \right) \text{Kernel density estimation} \quad (1)$$

$$K_i = \begin{cases} \frac{3}{4} (1 - x^2) & \text{Epanechnikov - Kernel} \\ 0 & \end{cases} \quad (2)$$

$$h = 0.9 \min \left( \hat{\sigma}, \frac{IQR}{1.34} \right) n^{-1/5} \text{SilvermanMethod} \quad (3)$$

$x$  = independent and identically distributed samples, IQR = interquartile range.

$\hat{\sigma}$  = standard deviation,  $h$  = bandwidth,  $K$  = kernel,  $\hat{f}_h(x)$  = kernel density estimation.

## 3 Results

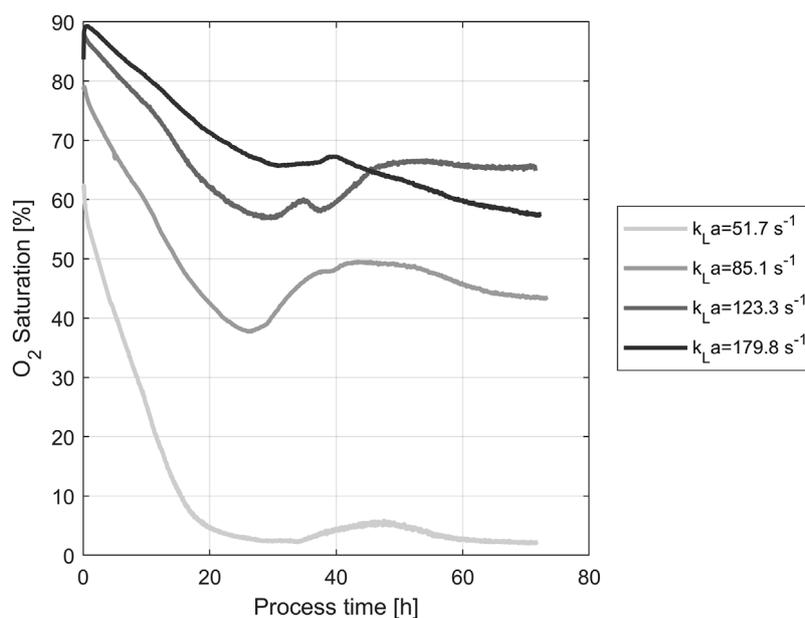
### 3.1 Oxygen saturation during propagation

DO concentration during yeast propagation in shake flasks was measured using noninvasive optical online sensors. The resulting concentrations for all four tested shaking speeds are shown in Figure 1. During the first hour post-inoculation, the oxygen content of the medium increased rapidly. The maximum of each curve depends on the respective  $k_{La}$ , generated through different shaking speeds. As the duration of the process increases, so do the cell count and the associated oxygen uptake rate. The lower the speed of the shaker, the less the increased oxygen demand of the cultures can be met. A rapid decline to nearly zero was shown after 30 h, especially in the oxygen saturation at 100 rpm shaking speed. The oxygen saturation of the medium was coupled with the shaking speed of the flasks. Therefore, oxygen saturation provided a reasonable basis for the following experiments.

### 3.2 Relative gene expression

#### 3.2.1 Melting curve analysis and determination of PCR amplification efficiency

The quality checks performed before gene expression analysis are shown in Figure 2. After extraction, RNA quality was tested using capillary gel electrophoresis (Figure 2A). The formation of two distinct bands (28S and 18S RNA) in the upper part of the gel indicated good RNA quality. Degradation of RNA would come with a significant band at the bottom of the gel and the absence or low intensity of the 28S and 18S bands. The capillary gel electrophoresis shows RIN values between 7.3 and 8.6, indicating reliable RNA quality and minor degradation (Schroeder et al., 2006). This quality is necessary for the following experiments. After testing the integrity of the RNA, all primers were tested by checking the specific primer bonding using agarose gel electrophoresis of the resulting amplicons. A representative gel of the primers tested is shown in Figure 2B.



**FIGURE 1**

O<sub>2</sub> Saturations in the medium during yeast propagation. The different shaking speeds correspond to the following  $k_L a$  values: 100 rpm: 51.7 s<sup>-1</sup>, 150 rpm: 85.1 s<sup>-1</sup>, 200 rpm: 123.3 s<sup>-1</sup>, 250 rpm: 179.8 s<sup>-1</sup>.

There was just one band indicating one size of amplicons per primer pair and a lack of bands for the NTC. As the last step, the amplicons were checked by melting curve analysis. The melting curve peaks are characteristic of a particular PCR product. The melting curve analysis served as a qualitative evaluation of the PCR product. The curve helps to compare and differentiate different PCR products concerning their melting behavior. All curves had a unimodal distribution proving specific primer binding to the target gene (Figure 2C) (Ririe et al., 1997).

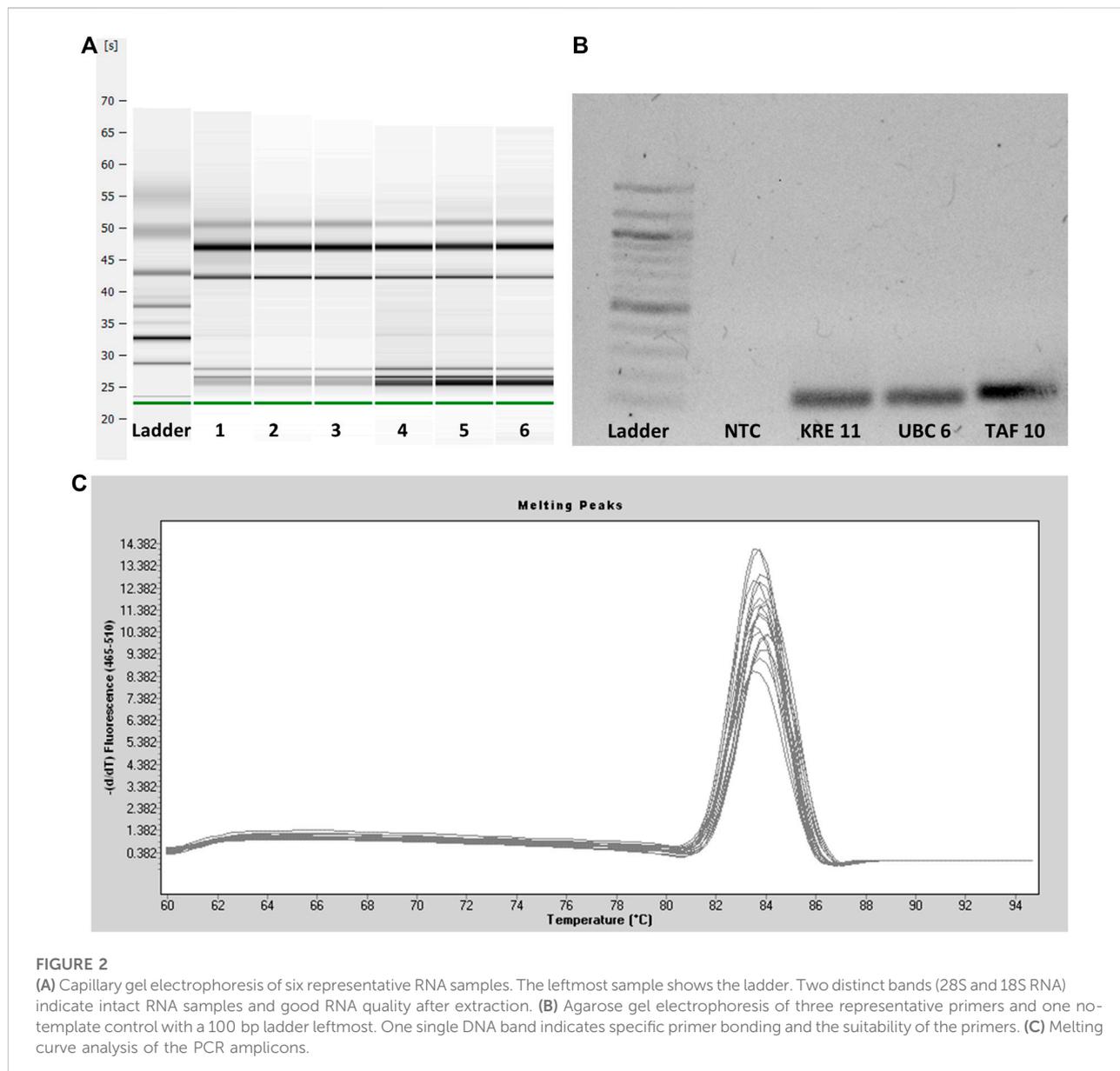
The amplification efficiencies (E) for the eight candidate reference genes ranged from 1.75 to 2.36, and standard errors (SD) were between 0.027 and 0.170 (Table 3). Agarose gel electrophoresis of all reference gene amplicons showed one single band, and the additional melting curve analysis revealed the presence of a single peak, indicating the specificity of the primers used. No signal was detected in the negative controls for all eight reference genes.

### 3.2.2 Reference gene system for *S. pastorianus*

The built-in geNorm algorithm of qBase Plus was used to calculate the M and V values and to evaluate the average expression stability of the reference genes. The M value is a measure used to determine the expression stability of control genes based on non-normalized expression levels. Unlike ideal reference genes, real genes are not consistently expressed under all experimental conditions. Variations in expression ratios indicate unstable expression of reference gene candidates. The M value defines the expression stability of all reference genes

based on the geometric averaging of multiple logarithmically transformed reference genes and their standard deviations during pairwise variation. In this method, the lowest M value correlates with the highest stability. Since the reference selection is based on non-core-regulated genes, stepwise exclusion of the most unstable gene results in two reference genes with the most stable expression. Stably expressed reference genes show M values lower than 0.5 for homogeneous samples. The last three tested reference gene candidates met these criteria (Vandesompele et al., 2002).

Normalization by multiple reference genes is required to increase measurement stability under different experimental conditions. The best reference gene combination always compromises accuracy and practical considerations. The pairwise variation is calculated between the two consecutive normalization factors  $NF_n$  and  $NF_{n+1}$  to assess whether the inclusion of more genes is helpful for normalization. Reference genes are added until the (n+1)<sup>th</sup> gene has no significant effect on the recalculated normalization factor. The value of  $V_{3/4}$ , displayed in Figure 3, is already under the threshold of 0.15 suggested by Vandesompele et al., below which the inclusion of additional reference genes is not required (Vandesompele et al., 2002). In the shown case, the inclusion of a fourth reference gene had no significant effect on normalization. Together with the M values shown in Figure 4, the resulting reference gene system for the following gene expression experiments consists of the genes KRE11, UBC6, and TAF10. Both quality criteria, the M value, and the V



value, are below the minima suggested by literature (Vandesompele et al., 2002), rendering the developed reference gene system suitable for the following analysis.

### 3.2.3 Influence of aeration on relative gene expression

The results of the CTA1 gene expression analysis for four different  $k_L a$  over the experimental time of 72 h relative to the developed reference gene system are shown in Figure 5: Relative expression of CTA1 gene during yeast propagation in shake flasks, including the 95% confidence interval. The data show increased expression of the CTA1 gene after 48 h. For the first 24 h, there was a low expression for all tested samples and shaking speeds. Since the

preculture was treated the same way for every sample, the 0- and 4-h samples were expected to be analogous. The 48-h samples showed a two- to 3-fold increase in expression compared to the 0-h sample. After 72 h, the 100 rpm sample had an even higher expression. The CTA1 gene tested in this experiment was responsible for the catalase production of the cell. This metal-dependent enzyme is part of the primary defense mechanisms against ROS. Catalase reduces the hydrogen peroxide content in the cell by reducing it using the dismutase mechanism (Toledano et al., 2003). Hydrogen peroxide is a product of oxygen reduction built as a by-product of cellular respiration. It enables the formation of other radicals in cells and is an essential part of reactive oxygen species. Due to the relationship between catalase and ROS, CTA1 expression should directly

TABLE 3 Amplification efficiencies and standard errors of designed primers.

Gene	Base for exponential amplification E	Standard error
YRB1	2.106	0.027
PGK1	1.954	0.027
ALG9	1.754	0.172
PFK1	2.340	0.080
TFC1	2.122	0.038
TAF10	2.158	0.041
UBC6	1.833	0.046
KRE11	1.880	0.041
CTA1	2.364	0.154

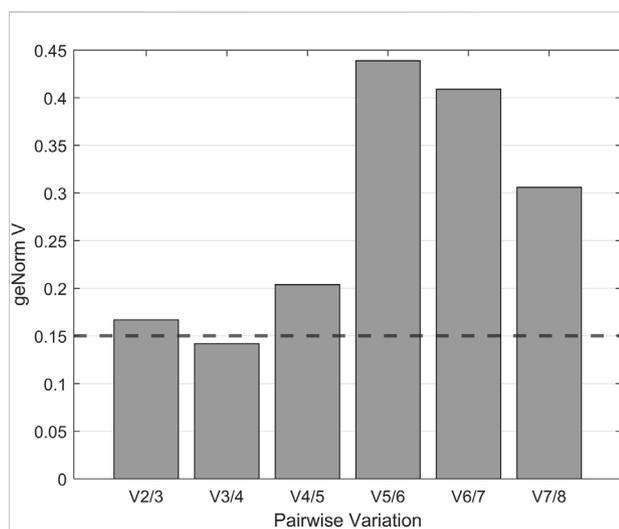


FIGURE 3

geNorm V analysis for optimal reference gene number determination. Pairwise variation was used to determine the minimum number of reference genes for normalization. According to Vandesompele et al. (2002) variations below the cut-off value of 0.15, displayed as a dashed horizontal line, indicate no significant contribution of an additional control gene to the normalization factor.

correlate with oxidative stress in the cell. Overall, there seems to be a trend in increasing CTA1 expression after 48 h, although there was no significant difference between the samples under different experimental conditions.

### 3.3 Determination of reactive oxygen species using flow cytometry

Applying the equations described in 2.3 to the flow cytometry data results in the graphs shown in Figure 6. It shows the triplicate

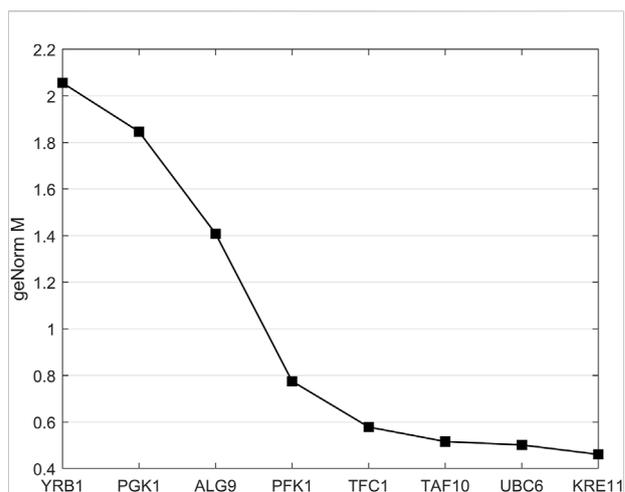


FIGURE 4

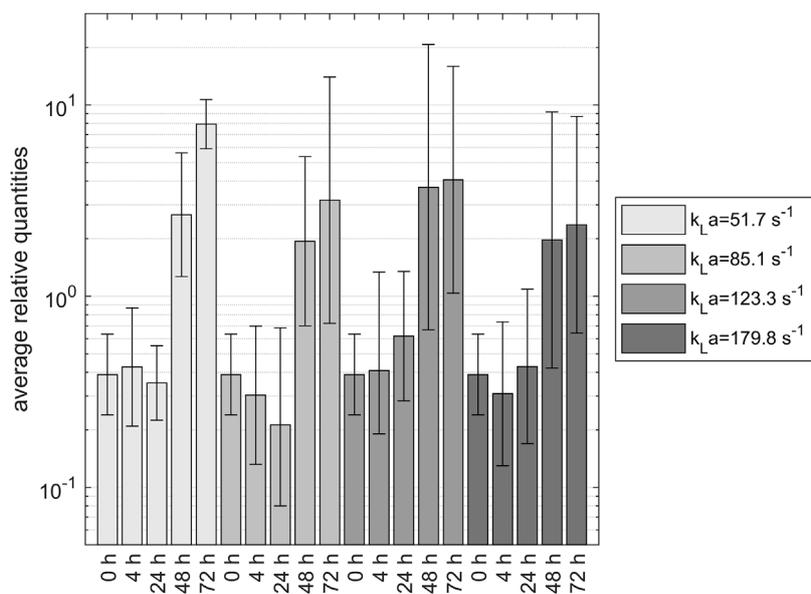
geNorm M analysis for analysis of reference gene stability. More stably expressed genes were positioned on the right side of the diagram and less stably expressed on the left side. Reference genes with mean stability values below 0.5 are considered to be stably expressed.

samples of the 200 rpm experiment over time. The 200 rpm samples were chosen because of their representative character for the other experiments that showed similar results. Dihydroethidium is oxidized in the cell by ROS superoxide and hydrogen peroxide. As a result, fluorescence can be measured at 610 nm. Fluorescence correlates directly with the amount of ROS in the cell. At the beginning of each experiment, the main portion of the investigated cells had a fluorescence below  $10^4$  procedure-defined units (p.d.u.). After 4 h, a trimodal distribution formed with peaks at  $10^5$  p.d.u. and approximately  $5 \cdot 10^6$  p.d.u. After 24 h, there was a huge shift toward a fluorescence of  $10^6$ . After 48 h, the fluorescence decreased again below  $10^6$ . After 72 h, the fluorescence was still decreasing.

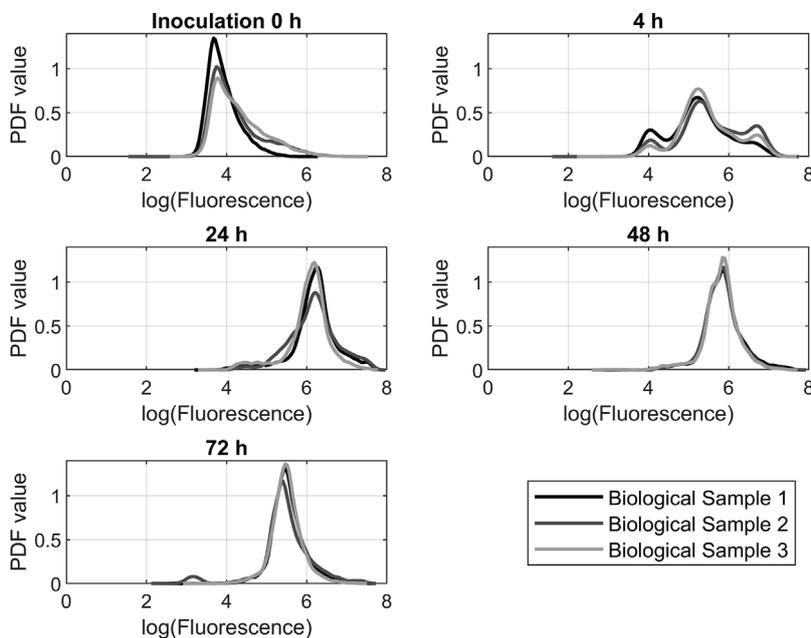
The logarithmic fluorescence of DHE for the 24-h samples of all tested shaking speeds is shown in Figure 7. The displayed samples are representative selections. The first measurement after inoculation was added as a comparative value with the fluorescence of approximately  $10^4$  p.d.u. The prominent peaks of the resulting graphs are around a value of  $10^6$  p.d.u. The 150 rpm and 200 rpm graphs had a unimodal distribution. At 100 rpm and 250 rpm, there was a bimodal distribution. For 100 rpm and 250 rpm, the distribution has a portion of cells with a fluorescence of  $10^4$  p.d.u., which is the reference value of the 0-h sample. This suggests that this portion of the cells did not react with the dye and could be excluded from further analysis. In this case, the remaining

## 4 Discussion

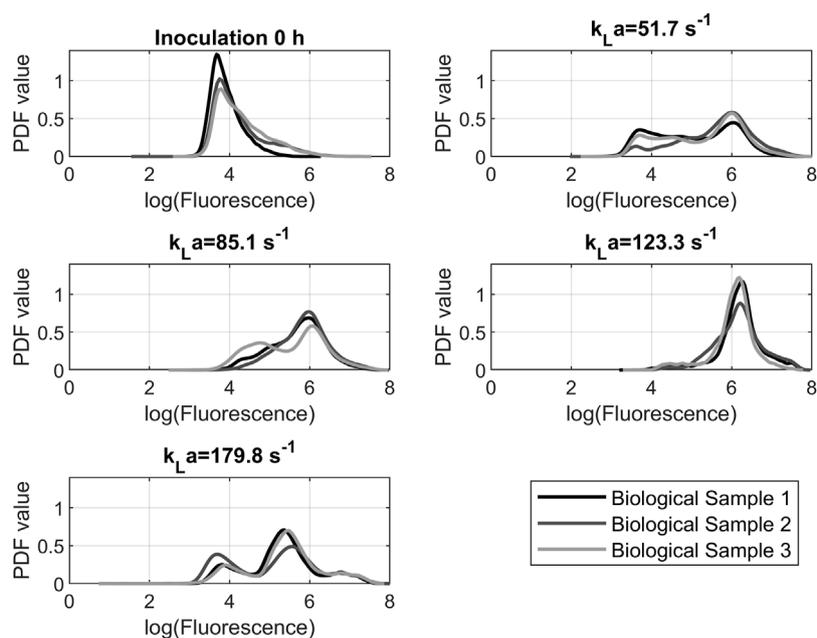
*Saccharomyces* is one of the most important industrially used yeasts and therefore needs to be supplied in a sufficient quantity



**FIGURE 5** Relative expression of CTA1 gene during yeast propagation in shake flasks, including the 95% confidence interval. The data show increased expression of the CTA1 gene after 48 h.



**FIGURE 6** Probability density function (PDF) of dihydroethidium fluorescence data from flow cytometry measurements. The data from the 200 rpm propagations was representative of the other experiments.



**FIGURE 7**

Probability density function of dihydroethidium fluorescence data from cytometry measurements after 24 h. The peak location indicates the probability that a cell will fluoresce at a certain level. The course of the graph indicates the distribution of fluorescence in the cell population.

and vitality. One of the key factors to efficient yeast propagation is oxygen supply to avoid growth limitations. Brewer's yeast *S. pastorianus* is a genetic hybrid of *S. cerevisiae* and *S. eubayanus* that shows high phenotypic and genomic similarities to *S. cerevisiae* and is highly relevant to industrial beer production. The presented study investigated the effect of aeration levels on oxidative stress response during the propagation of brewing yeast. For that purpose, a reference gene system for RT-qPCR analysis of *S. pastorianus* was developed: primers for an array of potential reference genes were screened for amplification efficiency and target expression stability under different aeration conditions. mRNA was successfully extracted from the cells and the subsequent quality check using capillary gel electrophoresis showed two distinct bands of 28S and 18S RNA and the absence of a strong lower band. The resulting bands indicate minor degradation of the extracted RNA. Additionally, capillary gel electrophoresis was utilized to determine the quality of the RNA. RIN values between 7.3 and 8.6 indicate suitable RNA quality for the following steps. Agarose gel electrophoresis was used to test specific primer bonding. The absence of multiple bands and a negative NTC prove the reliability of the synthesized cDNA. The unimodal form of the melting curves also indicates the specific primer bonding. The reference gene system was developed by using the geNorm algorithm. In a first step, all potential reference genes were sorted by stability calculating the geNorm M value. In a second step, the geNorm V value calculates the optimal number of reference genes. The three most stably

expressed genes were successfully utilized as reference for the transcription analysis of the CTA1 target gene in yeast samples propagated in lager beer wort. The CTA1 gene, coding for the enzyme catalase, that plays a major role in ROS degradation, was used to indicate oxidative stress in *S. pastorianus* cells under different aeration conditions. Intracellular reactive oxygen species were measured using flow cytometry with dihydroethidium staining. The resulting fluorescence at 610 nm correlates directly with the amount of ROS in the cells. In conjunction with the CTA1 transcription analysis, this measurement enabled a time-resolved insight into the build-up of oxidative stress and the cellular stress response. Comparing the results from the expression analysis with the results of the ROS determination, the ROS build-up took 24 h. The expression of CTA1 as a reaction of the cell to the increasing ROS content increased after 48 h. The higher CTA1 expression might be a reason for the decreasing ROS content, since catalase catalyzed ROS degradation; the genes expression appears to follow the increasing ROS content of the cell in a delayed fashion. Cells cultivated under low aerobic conditions showed a similar fluorescence behavior as those cultivated under the higher aeration efficiencies. Contrary to intuitive assumptions, the ROS built up independently of the shaking speed for the investigated conditions. This phenomenon follows the results of the gene expression analysis. In these experiments, increased CTA1 expression was shown after 48 h. The expression, however, like the fluorescence values, was independent of the cultivation

conditions. The presented data of gene expression analysis as an indicator for the cellular defence reaction of *Saccharomyces* yeast to higher aeration rates in combination with flow cytometry analysis as an indicator for the overall cell reaction can give useful information for later applications. An overall similar trend emerges between different samples propagated at different  $k_{1,a}$  values. This indicates that the aeration efficiency in the tested range does not provoke a cellular reaction. The tested oxygen supply conditions cover  $k_{1,a}$  values of industrial relevance; therefore, these results indicate the possible usage of higher oxygen concentrations in industrial yeast propagation. With this data, the study lays the foundation for further investigation and optimization of industrially relevant yeast propagation processes on a molecular level.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

## Author contributions

AB. Data collection and drafted the article, Conception or design of the work KB. Conception or design of the work DG.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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