JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Monitoring of Quorum-Sensing Molecules during Minifermentation Studies in Wine Yeast

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ABSTRACT: At high cell density or under low nutrient conditions, yeasts collectively adapt their metabolism by secreting aromatic alcohols in what is known as quorum sensing. However, the mechanisms and role of quorum sensing in yeast are poorly understood, and the methodology behind this process is not well established. This paper describes an effective approach to study quorum sensing in yeast fermentations. The separation, detection, and quantification of the putative quorum-sensing molecules 2-phenylethanol, tryptophol, and tyrosol have been optimized on a simple HPLC-based system. With the use of a phenyl HPLC column and a fluorescence detector, the sensitivity of the system was significantly increased. This allowed extraction and concentration procedures to be eliminated and the process to be scaled down to 2 mL minifermentations. Additionally, an innovative method for rapid viable-cell counting is presented. This study forms the basis for detailed studies in kinetics and regulation of quorum sensing in yeast fermentation.

KEYWORDS: *2-phenylethanol, HPLC, ImageJ, fermentation, quorum sensing, wine yeast*

INTRODUCTION

Quorum sensing (QS) is a process of intercellular communication. It allows individual cells to assess the population density and to coordinate their behavior by secreting small QS molecules into their environment.[1−3] Like the bacterium *Vibrio fisheri*,[4] the yeast *Candida albicans* was among the first fungi reported to have a QS system that controls the morphological switch between the cellular yeast form and the filamentous hyphal form when the cell density reaches the "quorum".[5] Two antagonistic QS molecules, farnesol and tyrosol [2-(4-hydroxyphenyl)ethanol], have been shown to inhibit and promote, respectively, the formation of germ tubes in *C. albicans*.[6−9] Similarly, *Saccharomyces cerevisiae* converts phenylalanine and tryptophan to the aromatic alcohols 2-phenylethanol and tryptophol [3-(2-hydroxyethyl)indole] via the Ehrlich pathway. 2-Phenylethanol and tryptophol have been reported to have QS roles under low nitrogen conditions, whereby they promote the shift of the cells into the stationary phase and regulate the induction of pseudohypha formation and invasive growth, according to the local cell density.[2,7,10−12] In addition to this biological role, the aromatic alcohols 2-phenylethanol, tryptophol, and tyrosol also have numerous biotechnological applications, especially in wine quality assessment,[13,14] aroma production in food and drinks,[15,16] and cosmetics,[17,18] and they can also act as antioxidants, antimicrobials, and/or disinfectants.[19]

Cells produce these QS molecules in relatively low concentrations (μM range). Therefore, to date, the use of the following separation and detection techniques has been reported: (i) inexpensive methods such as HPLC−diode-array detection[6,10,15,16] and gas chromatography−flame ionization detection,[18,20] which involves various procedures to concentrate the analyte, such as lyophilization and distillation;[10,21,22] or (ii) more expensive but more sensitive methods, such as gas or liquid chromatography−mass spectrometry.[13,14,23,33] However, in all of these cases, the extraction of the analyte is without exception an essential part of the protocol. Samples are usually extracted with organic solvents, such as ethyl acetate,[24] dichloromethane,[22,25] 1-hexanol,[26,27] and butyl acetate,[18] or by organic solvent mixtures, such as pentane/dichloromethane.[28] More recent extraction techniques also include solid phase extraction columns[6,13,14] and headspace solid phase microextraction.[20,23,29] Very little has been reported about the recoveries of the various extraction methods. In two studies found, the recoveries for the extraction with dichloromethane and solid phase extraction columns were in both cases >78%.[14,25] However, the extraction and concentration steps significantly extend these procedures and increase their complexity, in terms of the throughput and recovery of these methods. Moreover, to improve yields even more, medium volumes are kept relatively large (>50 mL),[6,14,15,22,26,33] which additionally lowers the throughput for these methods.

Along with the detection of QS molecules, the monitoring of cell concentrations is of great importance for the correct interpretation of QS. In most cases, a spectrophotometer is used for this purpose. However, to study QS kinetics, the amounts of QS molecules produced should be normalized to the number of cells that produce them. Therefore, the spectrophotometer, which is equally influenced by viable and nonviable cells, as well as by small particles, is not suitable for this purpose.

Received: July 31, 2012
Revised: February 1, 2013
Accepted: February 18, 2013
Published: February 18, 2013

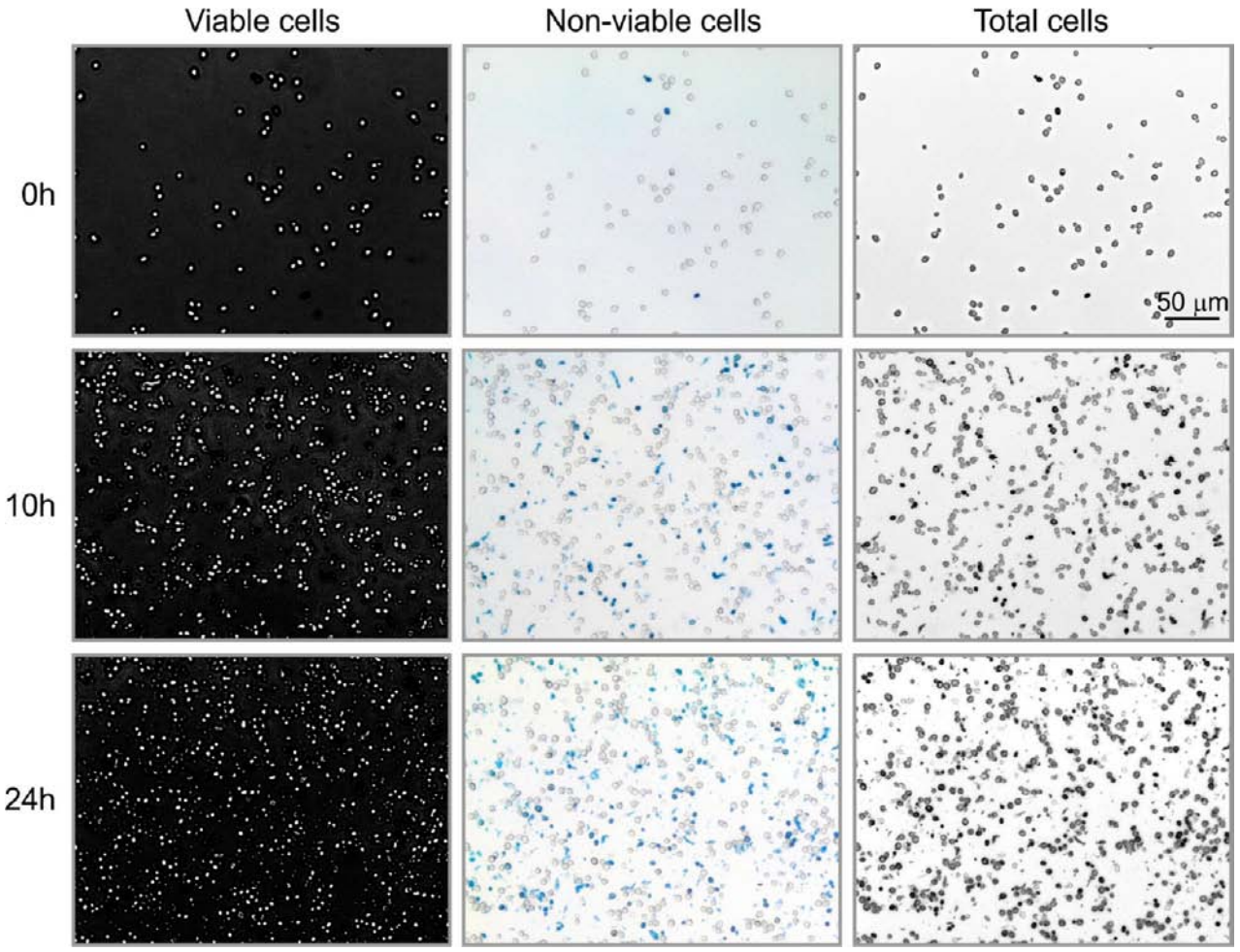

Figure 1. Automatic counting of viable, nonviable, and total cells with ImageJ (see also a movie in gif format available in the online version). Fermentation with *S. cerevisiae* was performed in 2 mL tubes containing MS300 medium with added magnetic stirrers at 22 °C for 28 h. Every 2 h, samples were taken for cell counting and HPLC analysis. Here, only three sampling times are shown; at the inoculation (0 h), at the peak of an exponential growth (10 h), and at the stationary phase (24 h). For counting of the viable, nonviable, and total cells, images were prepared with changes to the aperture, color, and gamma settings of a bright-field microscope connected to a camera (see Materials and Methods for details). The counting was performed in ImageJ for multiple images simultaneously, using protocols specific for each of the three groups of images. Only a portion of each original image size (ca. one-fourth) is presented here. Scale under 10× objective as shown.

To analyze as many samples as quickly and inexpensively as possible, we aimed to design a simple means of studying QS in yeast. According to current methodologies in this field, we noted three main drawbacks: (i) very low throughput of the methods, due to large fermentation volumes, which are a consequence of (ii) time-consuming and complex isolation procedures of QS molecules; and (iii) no knowledge of the QS kinetics during fermentation. We intended to scale-down the fermentation volumes and to avoid the extraction and concentration of QS molecules by using new phenyl HPLC columns and a sensitive fluorescence detector. To the best of our knowledge, such procedures have never been reported for the detection and quantification of 2-phenylethanol, tryptophol, or tyrosol in fermentation media. An additional aim was to provide the basis for the study of the kinetics of the production of QS molecules by replacing the use of a spectrophotometer with a method for the rapid determination of viable cell concentrations during the fermentation process.

MATERIALS AND METHODS

Strains and Inoculum Preparation. The ZIM 1927 *S. cerevisiae* wine yeast strain was used as a model organism for developing the platform, as it was originally isolated from the must of Malvasia wine grapes. To show the applicability of the methodology, other wine yeast species were tested as well: ZIM 842 *Candida zemplinina*, ZIM 701 *Dekkera bruxellensis*, ZIM 670 *Hanseniaspora uvarum*, ZIM 734 *Torulaspora pretoriensis*, and ZIM 850 *Zygosaccharomyces baili*, all five isolated from spontaneously fermented must of Rebula vine variety; and *S. cerevisiae* Lalvin EC1118 (Lallemand, Inc.), a starter culture for wine production. All of the strains were obtained from the Collection of Industrial Microorganisms (ZIM) at the Biotechnical Faculty, University of Ljubljana, Slovenia. These yeast strains were preserved in glycerol at −80 °C, and they were revitalized from frozen stocks by cultivation on YPD plates [YPD broth (Sigma, Germany), 2% bacteriological agar]. The inoculum was prepared in 100 mL of MS300 synthetic must as previously described,[30] with some modifications. The medium contained 100 g/L glucose and 100 g/L fructose, and no anaerobic factors were used. The inoculum preparation was performed for 48 h at 22 °C, with agitation at 220 rpm. After cultivation, the inoculum was centrifuged at 1500*g* and resuspended in 5 mL of fresh MS300 medium, to remove any QS molecules synthesized during the aerobic cultivation. The concentration of yeast cells was then determined as described below. The yeast suspension was diluted with MS300 medium to obtain a concentration of 2×10^7 cells/mL and then distributed into appropriate disposable fermenters.

Fermentation. A sample experiment was performed to show the four main aspects of the method's novelty acting together: (i) scale-down to minifermentations; (ii) automatic determination of viable yeast cell concentration; (iii) direct detection of QS molecules by HPLC with postcolumn fluorescence detection (HPLC-FLD); and (iv) kinetics of QS production. The fermentation was carried out under microaerobic conditions at 22 °C with added 10 mm magnetic stirrer bars if not stated differently, and the initial cell number was 2×10^7 cells/mL. The monitoring of the concentration of viable cells and QS molecules was performed at 2 h intervals during this 28 h fermentation procedure.

Scale-down. To test the possibility of replacing the Erlenmeyer flasks with smaller fermentation volumes, a scale-down approach was performed. For this purpose, preliminary fermentations were carried out in 2 mL microcentrifuge tubes (Eppendorf, Hamburg, Germany), 15 mL Falcon tubes (TPP, Switzerland), and 150 mL Erlenmeyer flasks, each containing 1.5, 10, and 100 mL of cell suspension, respectively. These were compared for cell densities and QS molecule concentrations. To enable CO_2 release, the microcentrifuge and Falcon tubes were sealed and perforated with a hot needle. For the flasks, the rubber stoppers were pierced with needles. Magnetic stirrers were used to agitate the fermentation broth, using 5, 10, and 25 mm magnetic stirring bars (Brand, Wertheim, Germany) in these 2, 15, and 100 mL fermenters, respectively. The fermentation tubes were agitated at 1200 rpm, whereas the agitation for the Erlenmeyer flasks was 500 rpm. To examine the need for this agitation during fermentation, no stirring was applied to a set of fermentation samples in the 2 mL microcentrifuge tubes. The cell concentrations for this comparative experiment were monitored at 0, 4.5, 8, 23, and 29 h, by collecting 20 μL of cell suspension from each fermenter. Viable cells were counted as described below. After 29 h, 1 mL of cell suspension was analyzed for QS molecules, using HPLC with postcolumn fluorescence detection (HPLC-FLD).

Automatic Counting of Viable Cells in Multiple Samples Using the ImageJ Software. In the present study, an innovative way of rapid image-based monitoring of viable yeast cells was used during fermentation. To show the accuracy of viable cell counting, the concentrations of viable, nonviable, and total cells were determined for three microcentrifuge tubes (representing a triplicate). In addition, 100 μL of cell suspension from each replicate was transferred to a microtiter plate to measure the optical density at 650 nm. The rest of the fermentation broth was further analyzed with HPLC for QS molecules as described in the next section. The concentrations of the cells were determined using methylene blue staining, a microscope with an attached camera (Leica DFC290), and ImageJ, v1.43u, image-processing software.[31] At each sampling point, 20 μL of cell suspension was collected from each replicate and diluted 1:1 (v/v) with methylene blue, which was prepared as defined previously.[32] The suspension was vortexed, and 20 μL of the stained suspension was then transferred into a 100 μm deep Bürker–Türk hemocytometer (BT, Brand, Wertheim, Germany). Three modes of microscopy were used, depending on which cells were to be counted: viable, nonviable, or total cells. These modes differed only in the settings of the microscope and were used successively with each preparation. When counting viable cells, the mode used was gray scale, bright-field microscopy with maximized gamma correction and maximally closed aperture, which resulted in light spots that represented viable cells on a dark background. Nonviable cells were counted using bright-field microscopy with maximally opened aperture and increased saturation, which revealed the blue-dyed nonviable cells. When total cell numbers were needed, the mode used was gray scale, bright-field microscopy with a half-closed aperture and increased gamma correction, which darkens the cells, resulting in black spots on a bright background (Figure 1).

The 10× magnification objective was used to cover as much area as possible, which enabled the counting of suspensions down to 2×10^5 cells/mL. Images were documented using Leica Application Suite, version 3.7.0 (Switzerland), and after the sampling, they were simultaneously analyzed using ImageJ, as follows: a set of images were loaded into the program, and then the threshold was adjusted to select for viable, nonviable, or total cells. The images were processed by the *Fill holes* and *Watershed* tools, to correct for weakly labeled cells and to separate buds, respectively. By using the *Analyze particles* tool of ImageJ, the labeled cells were counted selectively, considering their size and circularity, which reliably discarded any artifacts present in the sample (see also a movie in gif format available in the online version). The concentration of yeast cells in the inoculum or in the fermentation broth was then calculated, considering the volume according to the Bürker–Türk chamber depth (100 μm) and the surface covered by the microscope image (the volume here was 3.24×10^{-5} mL).

HPLC Monitoring of the Levels of the Quorum-Sensing Molecules. After cell counting, the remaining cell suspension (ca. 1 mL was enough) was prepared for HPLC analysis, with two consecutive centrifugations (at 1500*g* and 6500*g*) and with filtration through 0.2 μm filters (Phenomenex, Italy). HPLC analysis was performed using a reverse phase column (Waters, XBridge Phenyl, 5 μm, 4.6 mm × 150 mm), and the mobile phase was H_2O/acetonitrile 80:20 (v/v). The elution was isocratic, and the flow rate was 1 mL/min. The QS molecules 2-phenylethanol, tryptophol, and tyrosol were detected using a fluorescence detector (Shimadzu RF551, Japan), at their optimal wavelengths of 255/285, 280/368, and 275/315 nm, respectively. When the concentrations of these QS molecules were high enough, the detection of 2-phenylethanol and tyrosol was performed at 255/285 nm, and tryptophol was detected with a UV detector (Knauer VVM, Germany) at 280 nm.

For quantification of the concentrations of the QS molecules, standard calibration curves were constructed for 2-phenylethanol [≥99.0% (GC), Sigma-Aldrich, Germany], tryptophol [≥98% (GC), Fluka, Germany], and tyrosol [≥99.5% (GC), Sigma-Aldrich, Germany]. To evaluate the fermentation efficiency in the perforated 2 mL microcentrifuge tubes, the concentrations of ethanol, glucose, fructose, and acetic acid were determined by HPLC using an ion-exchange column (Bio-Rad Aminex, HPX 87H), a refractive index detector (Knauer, Germany), and 5 mM H_2SO_4 in water as the mobile phase at flow rate of 0.6 mL/min. The relevant standards for calibration curves were prepared with ethanol (Merck, for analysis, ACS, ISO, Reag. Ph Eur, 100%), glucose (anhydrous for biochemistry Reag. Ph Eur), fructose (for biochemistry), and acetic acid (for analysis, ACS, ISO, Reag. Ph Eur, 100%).

The HPLC-FLD quantification of these QS molecules was validated by the method of standard addition. Cultivation of the strain, 24 h of fermentation in 2 mL microcentrifuge tubes without agitation, and determination of the QS molecule concentrations were performed as described above. Successive amounts (1×, 2×, 3×, 4×, and 5 ×) of 2-phenylethanol, tryptophol, and tyrosol were then added into the tubes with the samples, and the QS molecule concentrations were measured again. The concentrations of the added standards were then plotted versus the total concentrations of the QS molecules in these samples. The amounts of the QS molecules produced during the fermentations were obtained by extending the calibration line to intersect the abscissa.

Quorum-Sensing Kinetics. The data from the measurements of cell and QS molecule concentrations were used to calculate the production rates of the QS molecules, providing new insight into the kinetics of QS molecule secretion.

Production Rate of Quorum-Sensing Molecules. The production rate of the QS molecules (R_{QSM}) between two time points was calculated as

$$R_{QSM} = \frac{(c_{QSM(t_2)} - c_{QSM(t_1)})}{\left(\frac{c_{t_1} + c_{t_2}}{2}\right) \times (t_2 - t_1)} \times 10^9 \text{ [fmol/cells/h]} \quad (1)$$

where t_1 and t_2 are the time points, c_{QSM} is the concentration of the QS molecule, and c is the cells/mL. To plot the R_{QSM}, curve fitting of the concentration of cells and QS molecules was first performed. The data from the fitted curves was then used in eq 1.

Average Production Rate of Quorum-Sensing Molecules. The information about the average production rate of QS molecules is useful when various cultivation conditions are compared and their influence on QS is studied. In addition, the number of samples and the

Table 1. Influence of Fermentation Volume and Agitation on the Average Production Rates of the QS Molecules

				av production rate of QS molecules (fmol/cells/h; ± SD)		
fermentation container	broth vol (mL)	agitation	(ΔetOH)/(Δ[glc + frc]) ± SD[a]	2-phenylethanol	tyrosol	tryptophol
microcentrifuge tube	1.5	no	1.7 ± 0.0	90.5 ± 5.4	22.8 ± 1.7	127.2 ± 1.9
microentrifuge tube	1.5	yes	1.7 ± 0.0	130.6 ± 5.7	36.4 ± 1.4	123.9 ± 5.4
Falcon tube	10.0	yes	1.9 ± 0.2	90.7 ± 9.7	18.4 ± 1.5	146.9 ± 23.3
Erlenmeyer flask	100.0	yes	1.8 ± 0.1	116.2 ± 3.6	23.4 ± 1.0	184.1 ± 5.2

[a]Moles of ethanol produced per mole of consumed glucose and fructose.

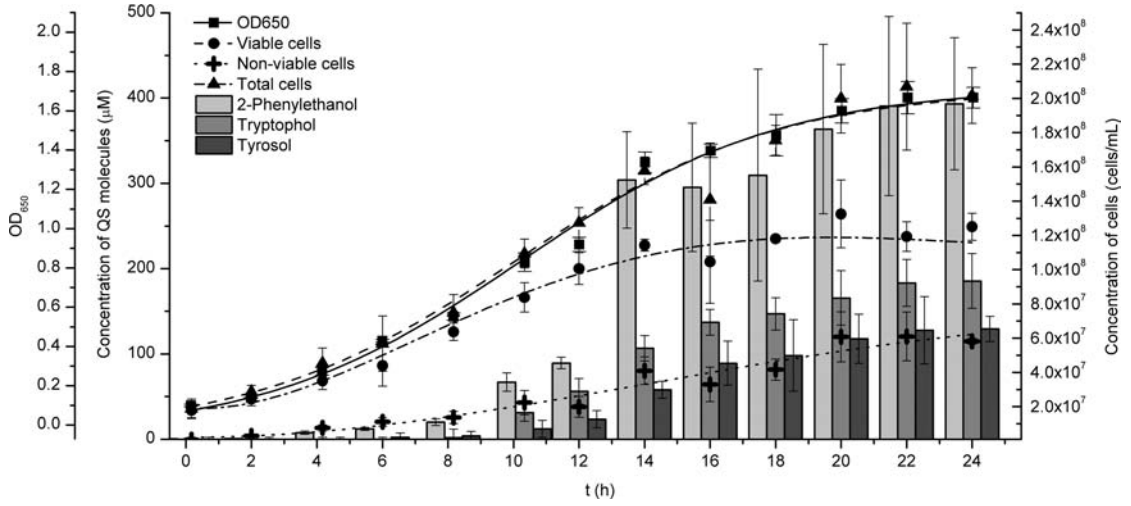

Figure 2. Comparison of the classical determination of cell concentrations using OD_{650} (solid squares with solid line) and ImageJ-based cell counting (broken lines) during the fermentation of *S. cerevisiae* in 2 mL tubes containing MS300 medium with added magnetic stirrers at 22 °C. The concentrations of the QS molecules are included in the histogram, as obtained using the proposed method. Data are the mean ± standard deviation of three independent replicates. Note that the total cell curve (solid triangles with dashed line) matches the OD_{650} curve and corresponds to the sum of the viable (solid circles wih dot-dashed line) and nonviable (crosses with dotted line) cell curves.

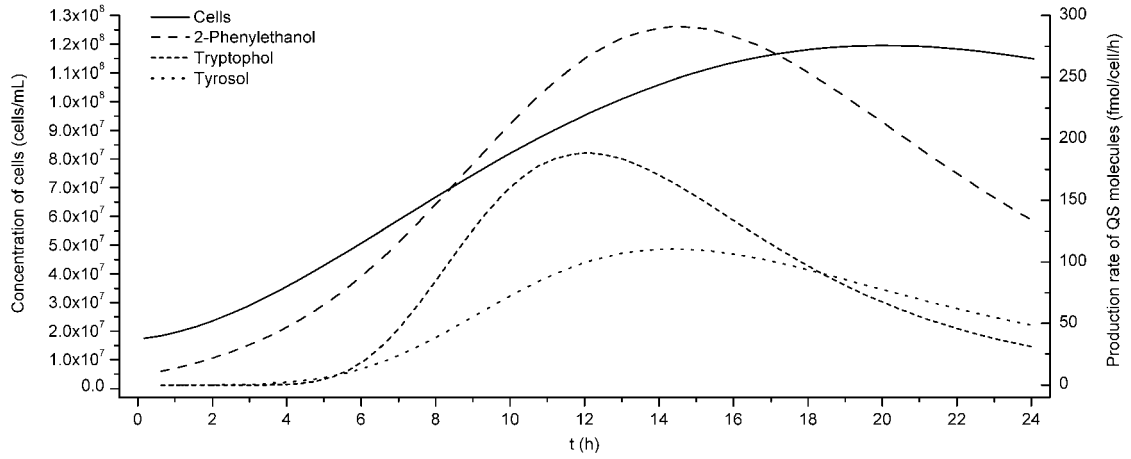

Figure 3. QS kinetics in *S. cerevisiae* during the fermentations in 2 mL tubes containing MS300 medium with added magnetic stirrers at 22 °C. Note that the production rates of the QS molecules (see eq 1) during the fermentation are calculated from the data presented in Figure 2. Data are the mean of three independent replicates.

amount of laboratory work are reduced, because the monitoring of QS molecules is not necessary in this case, except at the final point after fermentation. However, the shape of the growth curve is important; therefore, the measurements of viable cell numbers were performed five times: after 0, 4, 8, 24, and 29 h. The average production rates of QS molecules during the fermentation were calculated as

$$\bar{R}_{QSM} = \sum_{i=1}^{s-1} \frac{c_{QSM(t_s)}}{\left(\frac{c_i + c_{i+1}}{2}\right) \times (t_{i+1} - t_i)} \times 10^9 \text{ [fmol/cells/h]} \quad (2)$$

where t represents sampling time points, s is the number of sampling time points (here, as 5), c_{QSM} is the concentration of the QS molecule, and c_i is the concentration of cells at a specific sampling time point.

Production of QS Molecules in Other Yeast Species. After the optimization steps presented above, four wine yeast strains were analyzed using the proposed methodology. The fermentation was performed in 2 mL tubes containing MS300 medium at 22 °C for 28 h. At each sampling point, the concentrations of viable yeast cells and QS molecules were measured as described above. Finally, the kinetics of the production of QS molecules was studied on the basis of eq 1.

dx.doi.org/10.1021/jf3051363 | J. Agric. Food Chem. 2013, 61, 2496−2505

RESULTS AND DISCUSSION

Scale-down to Minifermentation. The idea was to carry out fermentations in small volumes, such as 2 mL microcentrifuge tubes and 15 mL Falcon tubes, to increase the throughput of the method. As a rule, broth volume is proportional to the efficiency of the extraction and/or detection procedures. The introduction of a sensitive fluorescence detector and the optimization of wavelengths for each QS molecule lowered the limits of detection (LODs) significantly, which allowed the extraction and concentration steps to be omitted. Indeed, when tryptophol was quantified, the LOD obtained with the UV detector was 628 ng/mL, whereas the LOD obtained with the fluorescence detector was 3.5 ng/mL, which represents a 180-fold difference. As no concentration step was needed, a scale-down was a reasonable measure.

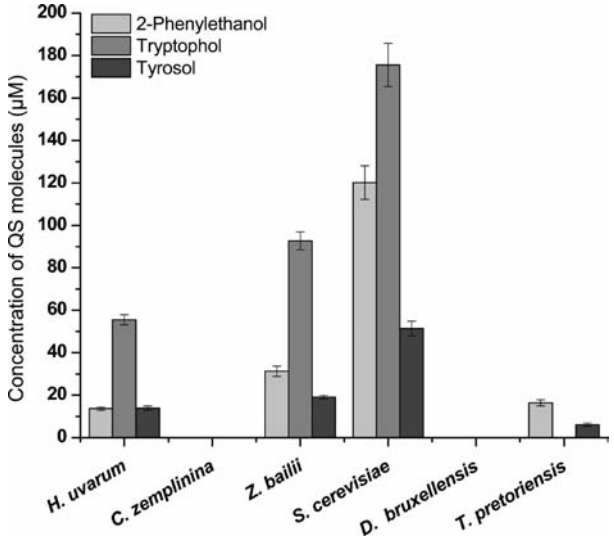

Figure 4. Production of QS molecules of other wine yeast species. The fermentation was performed in 2 mL tubes containing MS300 medium at 22 °C for 29 h. During the fermentation, concentration of cells was monitored, and at the end point the concentration of QS molecules was determined as described above.

Our results show that fermentation volumes as small as 1.5 mL can be used with no loss of reproducibility when compared to larger volumes (Table 1). Also, no general correlation was found between the fermentation volume and the concentration of aromatic alcohols produced (Table 1).

The necessity for agitation of samples was examined as well. The agitation stimulated the growth of the yeast (data not shown), and when agitation was used, the average production rates (see eq 2) of 2-phenylethanol and tyrosol were slightly higher, although the average production rate of tryptophol was not significantly different (Table 1).

To determine whether these scaled-down fermentation volumes and the perforation of the tubes still resulted in anaerobic yeast metabolism, that is, fermentation, we measured the ethanol production and the glucose/fructose consumption in these fermenters. To date, there are no experimental data on the effects of anaerobic growth conditions on 2-phenylethanol

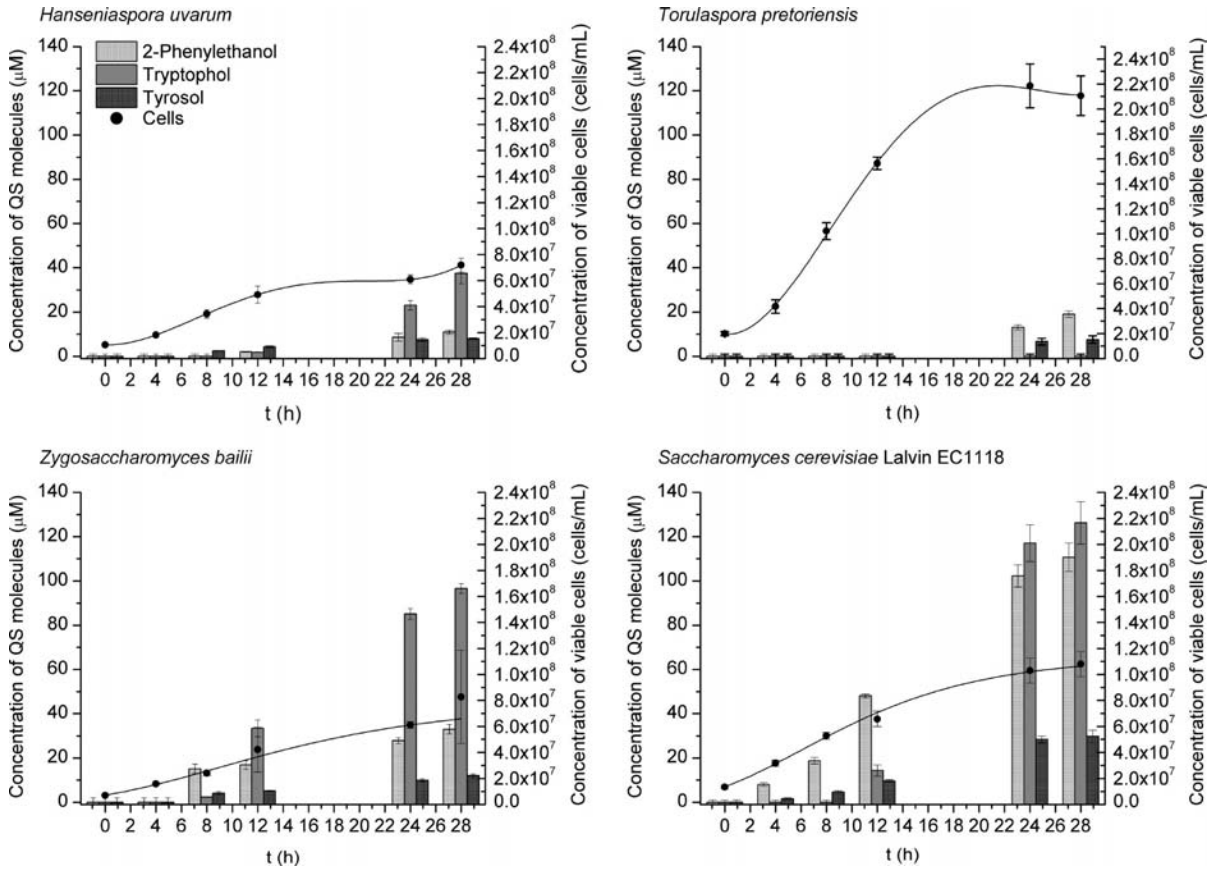

Figure 5. Monitoring of QS molecules and cell concentrations during the fermentations with four QS-active wine yeast species. The fermentations were performed in 2 mL tubes containing MS300 medium at 22 °C for 28 h.

dx.doi.org/10.1021/jf3051363 | J. Agric. Food Chem. 2013, 61, 2496−2505

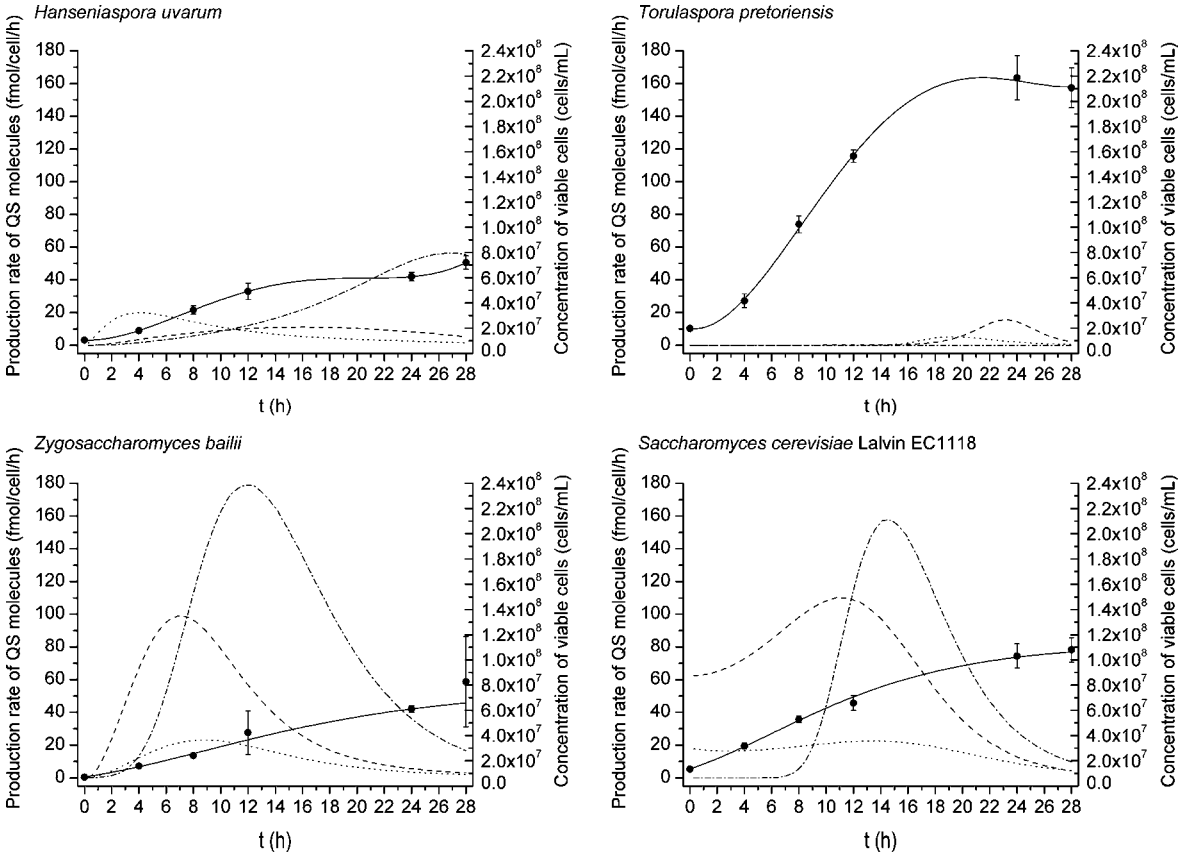

Figure 6. QS kinetics of four wine yeast species: cell concentration (solid line); 2-phenylethanol (dashed line); tryptophol (dash-dot line); tyrosol (dotted line). The fermentations were performed in 2 mL tubes containing MS300 medium at 22 °C for 28 h. Note that the production rates of the QS molecules (see eq 1) during the fermentations were calculated from the data presented in Figure 5.

Table 2. Validation Parameters for Aromatic Alcohol Detection

aromatic alcohol	excitation/emission (nm)	LOD[a] (ng/mL)	LOQ[b] (ng/mL)	linear range (μg/mL)	R^2
2-phenylethanol	255/285	750	2260	2−250[c]	0.9997
tryptophol	280/368	4	11	0.01−1.6[c]	0.9995
tyrosol	275/315	17	51	0.05−2.8[c]	0.9999

[a]LOD, limit of detection. [b]LOQ, limit of quantification. [c]If a UV detector is used instead of fluorescent detector, higher upper limits of linear ranges can be obtained.

and tryptophol production in *S. cerevisiae*. However, studies on *C. albicans* showed that increased aromatic alcohols are produced anaerobically and, moreover, that cells of *C. albicans* grown anaerobically at 30 °C produce roughly twice as much 2-phenylethanol, tryptophol, and tyrosol as they do aerobically.[24] We relied on the hypothesis that weak agitation and a Crabtree effect, which is the consequence of the high sugar content in the MS300 medium, would repress aerobic metabolism and favor fermentation. Indeed, the ratios between ethanol production and glucose/fructose consumption in the fermenters tested confirmed this hypothesis (Table 1).

The tubes were also checked visually during the fermentations, and no extensive sinking of cells was observed in the nonagitated tubes. The average coefficients of variation of the average production rate of these QS molecules were very low in all cases: 4% in the agitated 2 mL microcentrifuge tubes, 5% in the nonagitated 2 mL microcentrifuge tubes, 12% in the 15 mL Falcon tubes, and 3% in the Erlenmeyer flasks (Table 1). Considering all of the advantages of these 2 mL microcentrifuge tubes (i.e., low working costs, simplicity, among others) whether they were agitated or not, they proved to be highly suitable as minifermenters in these experimental studies of yeast QS.

Normalization Based on Viable Cell Counting by a Rapid ImageJ-Based Technique. It can be assumed that QS molecules are produced only by viable cells, and therefore a technique to determine the concentrations of the viable cells in the medium must be used. Optical density[10] and weighing of dry biomass[33] have often been used, although in both of these cases, nonviable cells and small particles are also included in these counts. Moreover, it is known that cell morphology, that is, cell length, has also a significant influence on the absorbance. Various commercial viability assays are also not suitable for this purpose, for several reasons, including they are time-consuming, very specific (e.g., they measure cell wall integrity, metabolic activity), and expensive. To obtain the closest approximation of the QS-producing yeast cell numbers, we optimized the counting of the viable cells in multiple samples using a hemocytometer and the freely available image-processing software ImageJ.[31]

During the 28 h fermentation with *S. cerevisiae*, described under Materials and Methods, samples were taken at 2 h

dx.doi.org/10.1021/jf3051363 | J. Agric. Food Chem. 2013, 61, 2496−2505

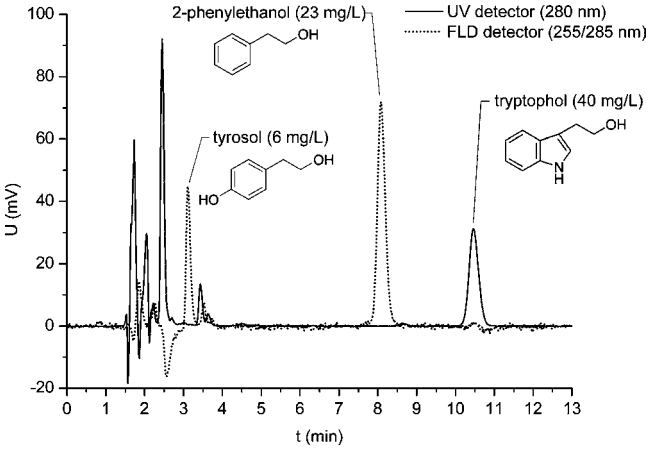

Figure 7. Representative HPLC chromatogram from a 2 mL fermenter sample, incubated for 29 h at 22 °C with no agitation. The detection of all three QSMs was performed simultaneously by UV and FLD detector. Note that only 2-phenylethanol is detected at its optimal wavelength. For improved LOD for tryptophol and tyrosol (see HPLC Monitoring of the Levels of the Quorum-Sensing Molecules), detection by FLD at 280/368 nm and 275/315 nm, respectively, can be performed.

intervals and the growth curves of viable, nonviable, and total cells measured using the ImageJ technique were compared with the growth curves obtained from the OD_{650} measurements. The results clearly confirmed that measurement via OD_{650} determines the total cell concentration, which is the sum of the viable and nonviable counts (Figure 2).

A possible explanation behind this technique is that viable cells can resist staining with methylene blue, and under specific settings of the microscope aperture, viable cells transmit light differently from nonviable cells, stained cells, particles, and other background. The result is light spots that are well separated on a dark background, which is a perfect sample for counting with the ImageJ algorithms. This software allows the collecting of the images in a specific directory, and then the analysis of all of the images simultaneously in one run (see Figure 1 and ImageJ, v1.43u, image-processing software for more details). This is very convenient for frequent sampling, which is of great importance with fast-growing microorganisms such as yeast.

This ImageJ-based counting was rapid (e.g., measurement of 10 samples can take 30 min from suspension to result), simple, and, above all, more informative, as the concentrations of viable, nonviable, and total cells are easily determined. In further experiments, only viable cells, which are important from the aspect of the production of QS molecules, were monitored in this manner.

Quorum-Sensing Kinetics during Fermentation. To better understand the QS mechanisms, the basis for the study of the QS kinetics was formed. In *S. cerevisiae*, the secretion of QS molecules was found to be related to the shift from exponential to stationary phase, when the induction of mechanisms needed for the starvation period occurs.[10,33] To study the influence of external factors on the production of these QS molecules and, consequently, to estimate the kinetics of these mechanisms, their production rates can be monitored through measuring their levels and the cell concentrations in the growth medium during the fermentation (see eq 1). The results from the minifermentation with ZIM 1927 *S. cerevisiae* strain (see Figures 1−3) clearly show that the production of all three of these QS molecules, 2-phenylethanol, tryptophol, and tyrosol, is most intense (i.e., the peak production rate) after the exponential phase, and then it steeply decreases before the onset of the stationary phase (Figure 3).

Chen and Fink[10] showed that the accumulation of 2-phenylethanol and tryptophol in the medium is growth phase dependent and reaches its highest levels after cultures enter into stationary phase. Studies by whole-genome microarray expression analysis have indicated that both of these aromatic alcohols regulate the transcription of a small set of genes, of which 70% are up-regulated upon entry into the stationary phase (e.g., ARO9 and ARO10, two key genes required for the biosynthesis of aromatic alcohols). Moreover, the production of aromatic alcohols is autostimulated by tryptophol. As tryptophol induces the enzymes required for its own synthesis, this provides a positive feedback loop. Therefore, cells at high population density produce more aromatic alcohols per cell than cells at low population density.[2,10] The induction of stationary phase expressed genes by aromatic alcohols suggests that these QS molecules might also have roles in signaling the correct entry of the *Saccharomyces* cells into the stationary phase.[2,10] These findings are in the agreement with the kinetics observed in the present study (Figure 3); the production rate of these QS molecules appears to reach its peak before the onset of the stationary phase, which could ensure that the released quorum-sensing molecules fulfill their function as molecular signals for entering cells into the stationary phase.

When the QS kinetics are not the aim of the experiment, such as if only the information about the average production rates of the QS molecules is needed, just one end point measurement of the concentrations of the QS molecules is sufficient. This kind of experiment was performed when different fermentation volumes were compared. In this way, the costs are significantly decreased, and the throughput of the assay is greatly enhanced. This thus gives an opportunity to perform more comprehensive studies, such as studies of the influence of numerous fermentation conditions on QS.

Production of Quorum-Sensing Molecules in Other Wine Yeast Species. The phenomenon of quorum sensing has mainly been examined in the yeasts *S. cerevisiae* and *C. albicans*. However, in our research we were interested in if and to what extent the following selected wine yeasts, *C. zemplinina*, *D. bruxellensis*, *H. uvarum*, *T. pretoriensis*, *Z. bailii*, and the commercialy used starter culture *S. cerevisiae* Lalvin EC1118, produce phenylethanol, tryptophol, and tyrosol. In this regard, independent minifermentation studies with the initial cell concentration of 2×10^7 cells/mL of the mentioned wine yeast strains were performed. In the first experiments with the end-point measurements of QS concentrations, it was shown that among the selected wine yeasts the yeasts *C. zemplinina* and *D. bruxellensis* do not produce any of the quorum-sensing molecules after 28 h of minifermentation (Figure 4).

To analyze the kinetics of QS molecules among the yeasts *H. uvarum*, *T. pretoriensis*, *Z. bailii*, and *S. cerevisiae* Lalvin EC1118, a second minifermentation experiment was performed. At each sampling point, the concentrations of viable cells and QS molecules were determined as described above. From the optimization experiments (Figures 1−3) we learned that the number of sampling points can be reduced to minimum five points, which still results in an accurate description of sigmoid growth curve and sufficiently supports quality interpretation; therefore, the sampling points in this case were at 4, 8, 12, 24, and 28 h, including the starting point at 0 h (Figure 5).

When suitable fittings of the growth curve and the QS molecule concentration curves are approximated, the kinetics of the production of these QS molecules can be obtained easily (see eq 1 and Figure 6).

The production rate of 2-phenylethanol and tyrosol reaches the highest value in the case of the commercial starter culture *S. cerevisiae* (Figure 6), which could have an important impact on the aroma profile of wine as the aromatic alcohol 2-phenylethanol is generaly a positive contributor to wine aroma. The yeast *Z. bailii* has the highest production rate of tryptophol and a kinetics of produced QS molecules similar to that of *S. cerevisiae*, which could be due to the fact that both species are assigned to the same family, Saccharomycetaceae, and exhibit therefore some phylogenetic similarity.[34] The yeast *H. uvarum* has a distinct kinetics of QS molecules; the production rate of tyrosol begins earlier and the production rate of tryptophol begins later when compared to *Z. bailii* and *S. bayanus*. Moreover, the production rate of phenylethanol is very low with no evident peak in our case (Figure 6). This heterogeneity of QS kinetics among the tested yeast species opens new questions about the roles of these aromatic alcohols in other species. In bacteria, it was recently shown that different quorum-sensing types or pherotypes exist even within the same species.[35] As mentioned in the Introduction, *C. albicans* "speaks a different language" when compared to the *S. cerevisiae* QS system; therefore, putative QS molecules in other yeast species still need to be discovered. The monitoring of the concentration and production rate of QS molecules opens a new and perspective area in wine fermentation. With the use of this platform, the strains for mixed wine fermentations could be better selected and suitably combined, which would improve the outcome of the end product.

Validation Parameters. To validate the method, the validation parameters of reproducibility, specificity, LOD, limit of quantification (LOQ), and linear range were determined. To validate the HPLC-FLD detection of the QS molecules, the method of standard addition was performed for all three of them.

Reproducibility. When we considered the bottleneck of this method regarding the reproducibility, the biggest influence on the method error was the organism, and not the method itself. When viable cells were counted, the average coefficient of variation in our experiments was 12%, but if six counts from the same fermenter were performed, a coefficient of variation of 5% was obtained. Similarly, the average coefficient of variation of the QS molecule concentrations for the HPLC analysis of samples was 8%, whereas for the QS molecule standards it was as low as 1%. This means that the handling of the samples should be as equal as possible, especially for the fermentation conditions and inoculation. We also noted that repeated dilutions of the yeast suspensions increased the error of the counting. We believe that the reason for this is respiratory metabolism and extensive production of CO_2 when the cell suspension is diluted and vortexed (the Crabtree effect is weakened, due to a decrease in the glucose and fructose concentrations). This will expand the volume of the suspension due to the formation of bubbles and result in an error each time a dilution is performed. For this reason, a not more than 2-fold dilution (methylene blue/suspension, 1:1, v/v) was used in all cases.

We also need to emphasize the importance of choosing the right filter for sample purification prior to HPLC analysis. When filters with regenerated cellulose were used, the recoveries of the QS molecules were always >95%, whereas in the case of polyvinylidene fluoride or polyethersulfone membrane filters, the recoveries dropped to 80 and 25%, respectively (data not shown).

Limit of Detection, Limit of Quantification, and Linear Range. The LOD for cell counting was determined as the cell concentration that results in only one cell being visible in the microscope image. Here, using the 10× objective, the lowest detectable concentration of cells was thus 6×10^4 cells/mL. However, the practical LOQ for the cell counting was at least 5-fold greater (i.e., 3×10^5 cells/mL). The LODs and LOQs for the HPLC analysis, together with linear ranges and R^2 of the calibration curves, are given in Table 2. The combination of a phenyl column and the HPLC-FLD system proved to be highly sensitive with low LOQs and a wide linear range. Moreover, when compared to similar publications, such as Gori et al.,[33] who also used a phenyl HPLC column for the separation of QS molecules, but with an MS/MS detector, some important observations can be made; their LOQs for phenylethanol, tryptophol, and tyrosol were 6.39, 4.84, and 2.75 μM, respectively, whereas in our case, the LOQs were 19 μM (3 times higher), 0.066 μM (73 times lower), and 0.36 μM (8 times lower), respectively. When the UV detector was used for the detection of tryptophol, the LOQ was comparable to the LOQ obtained by Gori et al. (11 μM).[33]

Specificity. The combination of the phenyl column (Waters, XBridge Phenyl, 5 μm, 4.6 mm × 150 mm) and the FLD/UV detectors was highly specific for QS molecule detection, as all three of the QS molecule peaks were clearly separate from each other and from the peaks of other medium components (Figure 7). Moreover, even the amino acid equivalents of these QS molecules, phenylalanine, tyrosine, and tryptophan, did not interfere with the QS molecules on the chromatograms: as standards, these were all detected in the first 3 min, specifically at 2.2 min for phenylalanine, 1.9 min for tyrosine, and 2.5 min for tryptophan. When compared with the HPLC-DAD systems, the specificity of the fluorescence detector is significantly greater, as the detected analytes must be sensitive to both the excitation and emission wavelengths to result in a peak.

The tests by standard addition confirmed the accuracy of the method. The measured concentrations of the QS molecules in the samples were 105.6 ± 0.9 μM for 2-phenylethanol, 223.8 ± 2.5 μM for tryptophol, and 20.2 ± 0.6 μM for tyrosol, whereas the theoretical concentrations obtained at the intersection of the calibration curves with the abscissa were 106.0 μM for 2-phenylethanol, 234.2 μM for tryptophol, and 19.7 μM for tyrosol; these represent 100, 105, and 98% of the measured values, respectively. The average coeficient of variation of the triplicates was 1%, and the average R^2 was 0.9999.

The optimizations of the different methods used in the present study resulted in the design of a rapid, simple, and inexpensive protocol for the study of QS in yeast. With the use of the new phenyl column and a fluorescence detector, we significantly increased the selectivity and sensitivity of the system. This allowed the elimination of extraction and concentration procedures and a scale-down of the process to these 2 mL minifermentations. In addition, we introduced an innovative way of rapid viable-cell counting, which enhances the interpretation of the data and enables detailed studies of the kinetics and regulation of these QS molecules in yeast fermentations. The protocol described here is actively used in our present studies, and here we show an example study of QS in other wine yeast species and the kinetics that lies behind this

process. We believe that this methodology will greatly contribute to the research field under discussion. The study of QS in yeast is a relatively "young" science, which needs new tools, and with this contribution, we have taken a step in this direction.

■ ASSOCIATED CONTENT

Ⓦ Web-Enhanced Features

Automatic cell counting of viable and nonviable yeast cells with ImageJ.

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Funding

This work was supported financially by the Slovenian Research Agency (ARRS).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Neža Čadež, the curator of the Collection of Industrial Microorganisms (ZIM) at the Biotechnical Faculty, Slovenia, for providing us with the yeast strains.

■ REFERENCES

(1) Fuqua, W. C.; Winans, S. C.; Greenberg, E. P. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J. Bacteriol.* **1994**, *176* (2), 269−275.

(2) Wuster, A.; Babu, M. M. Transcriptional control of the quorum sensing response in yeast. *Mol. Biosyst.* **2010**, *6* (1), 134−141.

(3) Atkinson, S.; Williams, P. Quorum sensing and social networking in the microbial world. *J. R. Soc. Interface* **2009**, *6* (40), 959−978.

(4) Nealson, K. H.; Platt, T.; Hastings, J. W. Cellular control of the synthesis and activity of the bacterial luminescent system. *J. Bacteriol.* **1970**, *104* (1), 313−322.

(5) Hornby, J. M.; Jensen, E. C.; Lisec, A. D.; Tasto, J. J.; Jahnke, B.; Shoemaker, R.; Dussault, P.; Nickerson, K. W. Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Appl. Environ. Microbiol.* **2001**, *67* (7), 2982−2992.

(6) Alem, M. A.; Oteef, M. D.; Flowers, T. H.; Douglas, L. J. Production of tyrosol by *Candida albicans* biofilms and its role in quorum sensing and biofilm development. *Eukaryotic Cell* **2006**, *5* (10), 1770−1779.

(7) Sprague, G. F., Jr.; Winans, S. C. Eukaryotes learn how to count: quorum sensing by yeast. *Genes Dev.* **2006**, *20* (9), 1045−1049.

(8) Nickerson, K. W.; Atkin, A. L.; Hornby, J. M. Quorum sensing in dimorphic fungi: farnesol and beyond. *Appl. Environ. Microbiol.* **2006**, *72* (6), 3805−3813.

(9) Kruppa, M. Quorum sensing and *Candida albicans*. *Mycoses* **2009**, *52* (1), 1−10.

(10) Chen, H.; Fink, G. R. Feedback control of morphogenesis in fungi by aromatic alcohols. *Genes Dev.* **2006**, *20* (9), 1150−1161.

(11) Hogan, D. A. Quorum sensing: alcohols in a social situation. *Curr. Biol.* **2006**, *16* (12), R457−R458.

(12) Leeder, A. C.; Palma-Guerrero, J.; Glass, N. L. The social network: deciphering fungal language. *Nat. Rev.: Microbiol.* **2011**, *9* (6), 440−451.

(13) Garde-Cerdán, T.; Marsellés-Fontanet, A. R.; Arias-Gil, M.; Ancín-Azpilicueta, C.; Martín-Belloso, O. Effect of storage conditions on the volatile composition of wines obtained from must stabilized by PEF during ageing without SO_2. *Innovative Food Sci. Emerging Technol.* **2008**, *9* (4), 469−476.

(14) Gonzalez-Marco, A.; Jimenez-Moreno, N.; Ancin-Azpilicueta, C. Influence of nutrients addition to nonlimited-in-nitrogen must on wine volatile composition. *J. Food Sci.* **2010**, *75* (4), S206−S211.

(15) Wang, H.; Dong, Q.; Guan, A.; Meng, C.; Shi, X.; Guo, Y. Synergistic inhibition effect of 2-phenylethanol and ethanol on bioproduction of natural 2-phenylethanol by *Saccharomyces cerevisiae* and process enhancement. *J. Biosci. Bioeng.* **2011**, *112* (1), 26−31.

(16) Etschmann, M. M.; Sell, D.; Schrader, J. Screening of yeasts for the production of the aroma compound 2-phenylethanol in a molasses-based medium. *Biotechnol. Lett.* **2003**, *25* (7), 531−536.

(17) Etschmann, M. M.; Schrader, J. An aqueous-organic two-phase bioprocess for efficient production of the natural aroma chemicals 2-phenylethanol and 2-phenylethylacetate with yeast. *Appl. Microbiol. Biotechnol.* **2006**, *71* (4), 440−443.

(18) Hua, D.; Lin, S.; Li, Y.; Chen, H.; Zhang, Z.; Du, Y.; Zhang, X.; Xu, P. Enhanced 2-phenylethanol production from L-phenylalanine via in situ product adsorption. *Biocatal. Biotransformation* **2010**, *28* (4), 259−266.

(19) Gañan, M.; Martínez-Rodríguez, J. A.; Carrascosa, V. A. Antimicrobial activity of phenolic compounds of wine against *Campylobacter jejuni*. *Food Control* **2009**, *20* (8), 739−742.

(20) Marti, M. P.; Mestres, M.; Sala, C.; Busto, O.; Guasch, J. Solid-phase microextraction and gas chromatography olfactometry analysis of successively diluted samples. A new approach of the aroma extract dilution analysis applied to the characterization of wine aroma. *J. Agric. Food Chem.* **2003**, *51* (27), 7861−7865.

(21) Chen, H.; Fujita, M.; Feng, Q.; Clardy, J.; Fink, G. R. Tyrosol is a quorum-sensing molecule in *Candida albicans*. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101* (14), 5048−5052.

(22) Kotseridis, Y.; Baumes, R. Identification of impact odorants in Bordeaux red grape juice, in the commercial yeast used for its fermentation, and in the produced wine. *J. Agric. Food Chem.* **2000**, *48* (2), 400−406.

(23) Martins, M.; Henriques, M.; Azeredo, J.; Rocha, S. M.; Coimbra, M. A.; Oliveira, R. *Candida* species extracellular alcohols: production and effect in sessile cells. *J. Basic Microbiol.* **2010**, *50* (Suppl.1), S89−S97.

(24) Ghosh, S.; Kebaara, B. W.; Atkin, A. L.; Nickerson, K. W. Regulation of aromatic alcohol production in *Candida albicans*. *Appl. Environ. Microbiol.* **2008**, *74* (23), 7211−7218.

(25) Fraile, P.; Garrido, J.; Ancin, C. Influence of a *Saccharomyces cerevisiae* selected strain in the volatile composition of rose wines. Evolution during fermentation. *J. Agric. Food Chem.* **2000**, *48* (5), 1789−1798.

(26) Bluemke, W.; Schrader, J. Integrated bioprocess for enhanced production of natural flavors and fragrances by *Ceratocystis moniliformis*. *Biomol. Eng.* **2001**, *17* (4−5), 137−142.

(27) Wittmann, C.; Hans, M.; Bluemke, W. Metabolic physiology of aroma-producing *Kluyveromyces marxianus*. *Yeast* **2002**, *19* (15), 1351−1363.

(28) Gueguen, Y.; Chemardin, P.; Pien, S.; Arnaud, A.; Galzy, P. Enhancement of aromatic quality of Muscat wine by the use of immobilized β-glucosidase. *J. Biotechnol.* **1997**, *55* (3), 151−156.

(29) Zhang, M.; Pan, Q.; Yan, G.; Duan, C. Using headspace solid phase micro-extraction for analysis of aromatic compounds during alcoholic fermentation of red wine. *Food Chem.* **2011**, *125* (2), 743−749.

(30) Bely, L.; Sablayrolles, J.; Barre, P. Description of alcoholic fermentation kinetics: its variability and significance. *Am. J. Enol. Vitic.* **1990**, *40*, 319−324.

(31) Rasband, W. S. *ImageJ*; U.S. National Institutes of Health: Bethesda, MD, 1997−2011; http://imagej.nih.gov/ij/.

(32) Painting, K.; Kirsop, B. A quick method for estimating the percentage of viable cells in a yeast population, using methylene blue staining. *World J. Microbiol. Biotechnol.* **1990**, *6* (3), 346−347.

(33) Gori, K.; Knudsen, P. B.; Nielsen, K. F.; Arneborg, N.; Jespersen, L. Alcohol-based quorum sensing plays a role in adhesion and sliding motility of the yeast *Debaryomyces hansenii*. *FEMS Yeast Res.* **2011**, *11* (8), 643−652.

(34) Kurtzman, C. P. Phylogenetic circumscription of *Saccharomyces*, *Kluyveromyces* and other members of the Saccharomycetaceae, and the proposal of the new genera *Lachancea*, *Nakaseomyces*, *Naumovia*, *Vanderwaltozyma* and *Zygotorulaspora*. *FEMS Yeast Res.* **2003**, *4* (3), 233−245.
(35) Stefanic, P.; Decorosi, F.; Viti, C.; Petito, J.; Cohan, F. M.; Mandic-Mulec, I. The quorum sensing diversity within and between ecotypes of *Bacillus subtilis*. *Environ. Microbiol.* **2012**, *14* (6), 1378−1389.