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The substratostat an automated near-infrared spectroscopy-based variable-feed system for fed-batch fermentations of grape musts

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Abstract

Aim: High sugar concentrations in musts cause a hyperosmotic stress response in *Saccharomyces cerevisiae* increasing the risk of sluggish and stuck alcoholic fermentations and/or causing high acetic acid levels. Applying a fed-batch technique where sugar levels are kept at a constant, low rate throughout fermentations reduces this stress but requires *in-situ* quantification of sugars and process automation for practicability. The aim of this work was to develop and validate a near-infrared (NIR) spectroscopy method allowing for the continuous *in-situ* quantification of total fermentable sugars in fully turbid alcoholic fermentations of grape musts. Calibration models for glucose, fructose and the fermentation product ethanol were also established.

Methods and results: A research Fourier-transform NIR spectrophotometer equipped with a transflectance probe was used to acquire spectra from 240 natural and semisynthetic standards from fermentations conducted using varying concentrations of yeast and yeast nutrients. Using chemometric software, calibration models for total sugars, glucose, fructose and ethanol demonstrated R^2 values > 0.93 and prediction error (RMSEP) values of 11.6 g l^{-1}, 12.3 g l^{-1}, 10.2 g l^{-1}, and 0.328 % v/v, respectively. The method was integrated with modern process automation technology and was able to maintain sugar concentrations within 5 g l^{-1} of the 45 g l^{-1} setpoint adjusted during alcoholic fermentations.

Conclusions: The NIR calibration models generated allow prediction of total sugar levels accurately enough to conduct fully automated fed-batch grape must fermentations at constant substrate concentrations. Application of a transflectance probe measuring a high proportion of back-scattered radiation proved useful and necessary considering the high degree of turbidity during fermentations. Placement of the measurement probe in a recirculation loop decreased interference from biomass sedimentation and adherence of CO_2 bubbles.

Significance and impact of the study: This study presents a fully automated system to carry out fed-batch fermentations which allow circumventing the hyperosmotic stress response of *S. cerevisiae* during alcoholic fermentations. Calibrated for other substrates, the system may be used in other food and non-food fermentations, too.

Key-words: wine, fermentation control, automation, yeast, near-infrared spectroscopy, in-line analysis

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Introduction

Microorganisms play a significant role in the production of numerous foods and beverages contributing to shelf-life, aroma, taste and nutritional properties (Doyle and Beuchat, 2007). High medium salt or sugar concentrations may lead to microbial stress response and adaptation or cellular inhibition and death (Grant, 2004). Such hyperosmotic conditions may be deliberately chosen during the food processing to enhance microbiological stability, or be naturally present in raw materials and affect the performance of production microorganisms. *Saccharomyces cerevisiae* is an acidophilic and ethanol tolerant production organism of preeminent importance for the production of foods and beverages, as well as modern biotechnological products. In hyperosmotic media containing significant sugar concentrations, such as grape must, *S. cerevisiae* displays a stress response that may result in the overproduction of several metabolites including acetic acid (Pigeau and Inglis, 2005a ; Pigeau and Inglis, 2005b) and acetaldehyde (Li and Mira de Orduña, 2011). In winemaking, the hyperosmotic stress response mediated metabolite profile of *S. cerevisiae* can be detrimental to wine quality and result in acetic acid levels exceeding legal limits.

Recently, Frohman and Mira de Orduña (2013) suggested a modified vinification protocol allowing to alleviate the yeast hyperosmotic response during the fermentation of grape musts containing high sugar concentrations (343 g l^{-1} combined glucose and fructose) that are encountered more frequently in hot climates or late harvest winemaking (Mira de Orduña, 2010). Instead of adding a yeast starter to the entire batch of grape must, the method consists of adding the grape must slowly to the yeast starter where the rate of must addition is adapted to the yeast metabolic rate in order to ensure that sugar concentration remains at a constant, low concentration. Application of this method allowed conducting fermentations of a high sugar containing must with increased yeast viability and a significantly reduced production of acetic acid (Frohman and Mira de Orduña, 2013). While providing proof-of-concept, the methodology applied was onerous requiring regular sample taking and sugar analysis, and manual adjustment of the must feeding rate, and clearly indicated the need for a continuous and automated *in-situ* quantification method. However, among the major challenges for an automated method are turbidity levels exceeding 10,000 NTU caused by yeast, nutrients and grape cell debris, effervescence from metabolic CO_2 release, temperature changes and large concentration gradients during fermentations.

Vibrational spectroscopy is a non-destructive and rapid measurement method with a flexible target compound range, not requiring sample taking or consumables nor being diffusion limited, and has been widely used in food analysis (Ozaki *et al.*, 2007). Specifically, near-infrared spectroscopy (NIRS) has been suggested for the analysis of unfiltered high turbidity media *in-situ* (Burns and Ciurczak, 2013). The purpose of the current work was to develop and validate a Fourier transform (FT)-NIRS method allowing for the continuous *in-situ* quantification of total fermentable sugars (defined as glucose + fructose) in fully turbid alcoholic fermentations of grape musts. The method was integrated with modern process automation technology enabling fully automatic grape must fermentations at constant substrate concentrations. Calibration models for glucose, fructose and the fermentation product ethanol were also established.

Materials and methods

1. Media, yeast and fermentations

Flash-pasteurized Chardonnay must from the Languedoc region of France (Kamil Juices, Canada) and unpasteurized Cabernet Franc must obtained from directly pressed grapes (Cornell University Vineyards, Geneva, NY) were utilized. High and low sugar containing musts were prepared by chaptalization with equal quantities of anhydrous D-glucose and D-fructose and/or by diluting with ASTM Class I water (Arium 611UV, Sartorius, Germany) and then fermented to generate samples for model generation validation. To further extend the range of sugar and ethanol concentration ratios and turbidity levels, additional samples were prepared by sterile filtering (0.22 µm, nylon, Millipore, Ireland) samples from such fermentations, followed by spiking with water, glucose, fructose, absolute ethanol, and yeast nutrients. Such samples were also saturated with CO_2 to imitate fermentation conditions. To prevent collinearity problems between sugar and ethanol concentrations in the models (Conzen, 2006), additional standards were created by removing ethanol from fermentation samples by rotary evaporation (Rotavapor R-200, Büchi, Switzerland). The volume thus removed was replaced with water. The dealcoholized wines thus obtained were then spiked with varying quantities of glucose, fructose and ethanol in order to create additional semisynthetic high sugar/high alcohol standards, as well as low sugar/low alcohol standards. In order to

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generate robust chemometric models, the concentration ranges of various fermentation parameters were adjusted to cover large concentration ranges : total sugars, 0-368 g l^{-1} ; ethanol, 0-21 % (v/v) ; yeast inoculum, 40-1 200 g hl^{-1} ; turbidity, 173-25,000 NTU.

Active dry *S. cerevisiae* strain EC1118 (Lallemand, Canada) was used for all fermentations and prepared according to manufacturer's recommendations by rehydrating for 15 minutes at 40 °C in ASTM Class I water (Arium 611UV, Sartorius, Germany). The yeast inoculation rate varied from 40 to 1 200 g hl^{-1} depending on the start volume, which is small in fed-batch fermentations (see below). For yeast nutrition, a complex supplement (Fermaid K, Lallemand, Canada) and $(NH_4)_2HPO_3$ were added to all musts at 0.25 g l^{-1}.

All batch fermentations were conducted isothermally (20 °C) by adding the rehydrated yeast starter to the entire amount of must in 2 l glass bottles (Kimble Chase, NJ) sealed with suitable air locks (Buon Vino Manufacturing, Canada) to allow for fermentation gas release and to prevent air ingress. These fermentations were inoculated with yeast at 40 g hl^{-1} with respect to the starting volume. Models generated with these initial batch-fermentations were used to carry out further training fermentations that were conducted in automated fed-batch mode in 28 l cylindroconical stainless steel tanks (Glacier Tanks, Oregon) at 20 °C.

For these fed-batch fermentations, the rehydrated yeast slurry was first added to the fermentation tank (Figure 1). Immediately after the rehydration period, must warmed up to 20 °C was manually added to reach a suitable start sugar concentration. Subsequently, the automation system was activated and maintained the total sugars at setpoint concentrations by adding must from the cooled must tank (Figure 1) until all must was consumed. After depletion of the must in the storage tank, the fermentations entered a batch phase until all sugar was consumed. Fermentations were conducted at 20 °C and were regarded as finished when the sugar consumption was less than 0.5 g l^{-1} in 24 hours.

2. FT-NIRS analysis, system automation and logging

The NIR analyser system for spectral acquisition and processing consisted of a FT-NIR spectrophotometer (MPA, Bruker Optics, Germany) equipped with a high-sensitivity InGaAs detector with a 12,500-4,000 cm^{-1} detection range, a stainless steel (SS316) and autoclavable process control transflectance probe (IN271F, Bruker Optics, Germany) with a 2 mm fixed optical path length (1 mm slit) and sapphire window (Figure 1B), and a 5 m fiber optic quartz cable with 7 fibers (IN226-05, Bruker Optics, Germany). The system was controlled using the acquisition, quantification and processing software provided (OPUS, Bruker Optics, Germany). All scans were acquired over the entire spectral range at a resolution of 16 cm^{-1}, and consisted of 1 minute of consecutive scans (~ 700 scans), which were subsequently averaged. Sample and preamp signal gain settings were set to X1 and X30, respectively, and the zerofilling factor was set to 8. Sample spectra were referenced against an air background that was collected immediately prior to the start of fermentations. The generation of calibration models and model validation is described in the results section and was performed with the chemometric model development platform of the FT-NIR equipment software package (QUANT module, OPUS v7, Bruker Optics, Germany). For batch fermentations, the transflectance probe was manually submerged into the fermenting liquid at regular intervals to acquire absorbance spectra. For automated fed-batch fermentations, the transflectance probe (« NIR » in Figure 1A) was placed in an external loop in which fermentation liquid was continuously recirculated by means of a peristaltic pump (704 U/R, Watson-Marlow, England) at a flow rate of 1 l min^{-1}. Following spectral acquisition and averaging, the total sugar values predicted by the model in the FT-NIR software (OPUS, Bruker Optics, Germany) were written to an OPC-server (OPC Server, Advantech, CA) and transferred via RS232 to a programmable logic controller (ADAM-5000/485, Advantech, CA) equipped with a four-channel analogue output module (ADAM-5024, Advantech, CA) capable of generating a 4-20 mA signal scaled to the total sugar concentration measured. A PID-controller (2216e, Invensys Eurotherm USA, VA) was used to compare the process variable (total sugar measured) with the setpoint value and to control the delivery rate of a programmable peristaltic pump (Masterflex L/S, Cole-Parmer, IL) feeding fresh grape must into the external loop. The auto-tune function of the controller was used to identify and apply the appropriate control parameters. For accurate temperature control, a temperature transmitter (3-wire PT100-RTD, ProSense, Automation Direct, GA) was installed in the external recirculation loop (« TT » in Figure 1A) and connected to a second PID-controller which controlled the positioning of a modulating electrically actuated ball valve (BI-TORQ, IL, USA) circulating cooling water (15 °C)

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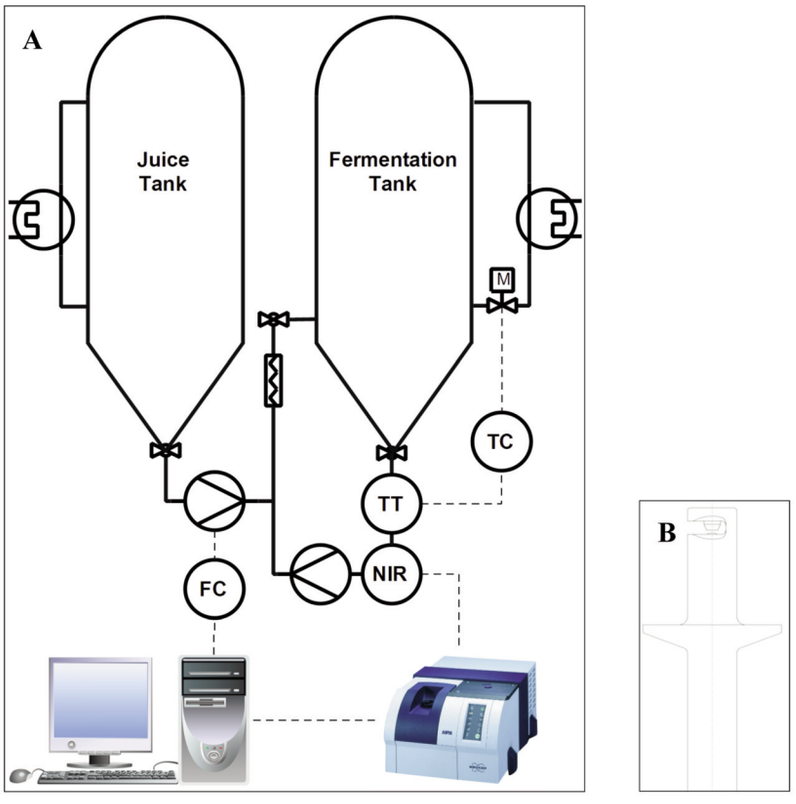


Figure 1. The substratostat – an automated continuous fed-batch fermentation system
A. Automated continuous fed-batch fermentation system. Thick solid lines indicate liquid flow; thin dashed lines indicate data flow. TT, temperature transmitter; TC, temperature controller; NIR, NIR-transflectance probe; FC, flow controller. B. Detail drawing of fixed optical path-length transflectance probe (IN271F).

through a stainless steel cylindroconical coil installed inside the fermentation tank. To inhibit microbial growth in the must prior to fermentation, the must tank was maintained at -2 to 0 °C by circulating water through a similarly designed coil. The substratostat system thus engineered is detailed in Figure 1.

3. Sampling and reference analyses

During all fermentations samples were taken under a constant stream of nitrogen to prevent air ingress and sample oxidation. After separation of the biomass by centrifugation (5 minutes, 15,000 *g*) the samples were immediately frozen at -18 °C for subsequent HPLC analysis. A high pressure liquid chromatography system (Shimadzu, Japan) consisting of a binary LC-20AB pumping unit, a DGU-20A3 degasser, a SIL-10AD VP autosampler, a CTO-20AC column oven, a SPD-M20A diode array detector, and a RID-10A refractive index detector was used for isocratic separation and analysis of sugars and ethanol. Data acquisition and analysis was performed with the instrument software provided (LCSolution v.1.23). The mobile phase consisted of ASTM Class I water with 1 % (w/v) HPLC grade phosphoric acid and 5 % (v/v) HPLC grade acetonitrile and was filtered prior to utilization (0.22 µm, nylon, Millipore, Ireland). After sample injection (5 µl), separation occurred at a flow rate of 0.35 ml min^{-1} on a sulfonated polystyrene/divinyl benzene stationary phase with 9 µm particle size (250 x 4.6 mm, Supelcogel H, Sigma Aldrich, MO) with a corresponding 50 x 4.6 mm guard column (Supelguard C610H, Sigma Aldrich, MO), both of which were held at 60 °C (Frohman and Mira de Orduña, 2013). Sugars and ethanol were quantified by refractive index. Both analytes were quantified using external calibration standards. Eight standards were utilized for each calibration curve. Glucose and fructose standard concentrations ranged from 1 to 200 g l^{-1}, and ethanol standard concentrations ranged from 0.5 to 20 % (v/v).

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Turbidity was quantified in must and fermenting wines with a turbidimeter (Ratio XR, Hach, USA) previously standardized with formazin standards (StablCal, Hach, USA).

4. Replication and statistical analysis

All fermentations were conducted in duplicate. The chemometric models were developed using the software package of the FT-NIR equipment (OPUS v7, Bruker Optics, Germany). Origin 9.0 Pro (OriginLab, MA, USA) was used for graphical representations.

Results

Separate partial least squares (PLS) models were generated for the prediction of total sugars (glucose and fructose), glucose, fructose and ethanol based on the FT-NIR spectra of approximately 240 authentic and semisynthetic samples generated by spiking grape must samples, or taken during the course of five training batch fermentations and three training fed-batch fermentations. Figure 2A displays a representative set of the absorbance spectra collected.

The sample spectra demonstrated a wide range of baseline absorbances (-1 to 1.8, Figure 2A) that was associated with sample turbidity caused primarily by suspended yeast. Vector normalization (SNV) was used as spectral pre-processing method (Figure 2B), except for the fructose model, where the first derivative was also used in combination with SNV in order to enhance the calibrations model (Table 1). Because of high inherent spectral noise, the wavenumber regions below 5000 cm^{-1} and above 11,100 cm^{-1} were excluded for all models in addition to the two predominant water absorption bands in the NIR spectral region, which are located in the 6600-7100 cm^{-1} and 4800-5300 cm^{-1} regions (Figure 2C). A summary of parameters for pre-processing and

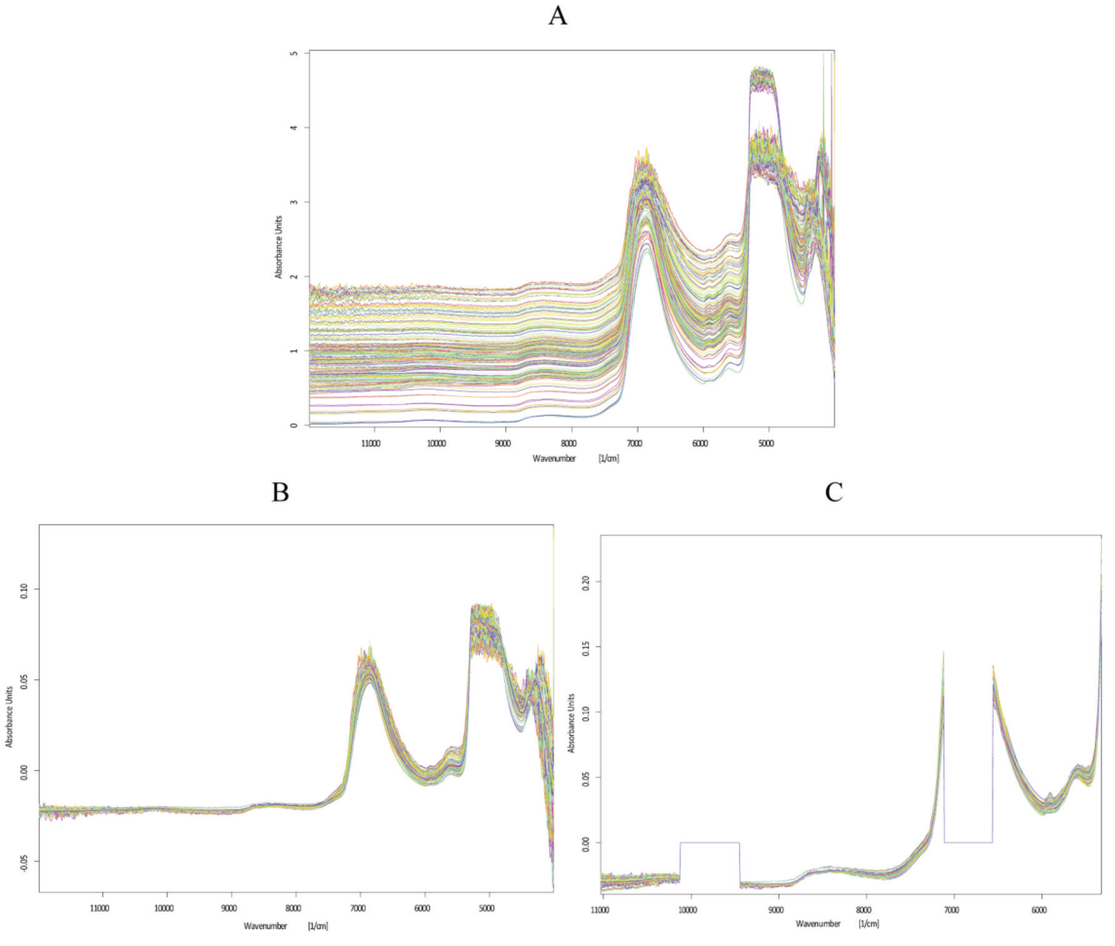


Figure 2. Spectral acquisition and processing
Sample spectra before pre-processing (A), following vector normalization (B) and after definition of spectral regions for prediction of total sugars (C). Empty regions were excluded from the model.

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Table 1. FT-NIRS calibration model pre-processing technique and calibration regions

Model	Pre-processing	Calibration Regions (cm^{-1})
Glucose	SNV	10229.4 – 9388.5
		8709.7 – 8115.6
		7683.6 – 7120.5
		6557.3 – 5323.0
Fructose	SNV	11031.7 – 7120.5
	1st Derivative	6557.3 – 5647.0
Total Sugars	SNV	11031.7 – 10136.8
		9442.5 – 7120.5
		6557.3 – 5323.0
Ethanol	SNV	9442.5 – 7621.9
		6557.3 – 5647.0

Table 2. Statistical parameters for final FT-NIRS calibration models

Model	N	Rank	R^2	RPD	RMSECV	RMSEP
Glucose (g l^{-1})	235	8	93.17	3.83	10.1	12.3
Fructose (g l^{-1})	236	7	93.25	3.86	10.7	10.2
Total Sugars (g l^{-1})	242	8	97.23	6.01	13	11.6
Ethanol (% v/v)	236	4	98.80	9.14	0.534	0.328

(RMSECV, root mean square error of cross validation; RMSEP, root mean square error of prediction)

spectral regions for the different calibration models is detailed in Table 1.

After definition of these limitations, a PCA factorization algorithm was used to identify and exclude redundant spectra, and to adjust the wavenumber intervals, number of factors (rank), and pre-treatment method so as to minimize the root mean square error of cross validation (RMSECV). The model rank was then manually readjusted to reduce noise, and model outliers were identified using a graphical representation of Mahalanobis distance versus spectral residue and removed from the calibration. The models thus adjusted were then tested against an external validation set consisting of approximately 30 randomly selected samples (excluded from calibrations), and the best performing model (lowest root mean square error of prediction or RMSEP) for each analyte was retained. As spectra from samples of additional training fermentations were obtained, all calibration models were progressively expanded and revalidated. Figure 3 shows the progression of the RMSEP of total sugars with increasing calibration size. The initial total sugar model which consisted of only 33 spectra performed poorly, yielding an RMSEP > 25 g l^{-1}. Doubling the number of standards drastically improved the RMSEP, decreasing it to 18 g l^{-1}. The addition of standards from subsequent training fermentations resulted in a final RMSEP of 11.6 g l^{-1} (Figure 3).

Graphical representations of the results of the final model calibrations and external validations for the prediction of total sugars, glucose, fructose and ethanol are provided with the Supplementary Information (Figure S1). Cross- and external validation statistics for the models are also summarized in Table 2. The model for the prediction of ethanol utilized a much lower rank (4), and yet exhibited the highest R^2 and RPD values (Table 2).

The three sugar models exhibited RMSECV and RMSEP values of approximately 10 g l^{-1}. A detailed analysis of the prediction errors of the final total sugar model according to the concentration range (Figure 4) showed that the error was greatest towards the lower (0-30 g l^{-1}) and higher (181-230 g l^{-1}) ends

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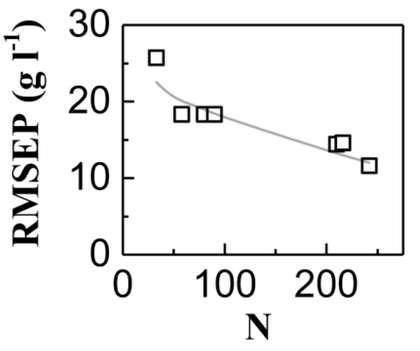


Figure 3. The effect of the number of training standards (N) on RMSEP (root mean square error of prediction) for the total sugar model (tested with external validation sets)

of the measurement range and smallest in the middle (121-150 g l^{-1}).

The performance of the initial (N = 33) and the final (N = 242) total sugar model (Table 2) was compared by conducting automated fed-batch validation fermentations with a setpoint of 45 g l^{-1} total sugars. This setpoint was chosen to test the system at average prediction errors (Figure 4) and because previous research had shown that sugar concentrations ≤ 50 g l^{-1} led to efficient reduction of yeast hyperosmotic stress response (Frohman and Mira de Orduña, 2013). Figure 5 shows the course of the fermentations for the initial model. After the initial increase of the sugar concentration by manually adding must, the fermentation entered the automated fed-batch phase which lasted until t = 250 heures At this time point, must in the storage tank was depleted and the fermentation entered a batch phase until all sugars were consumed resulting in a dry wine. However, due to the low number of utilized training standards (N = 33) and the high model RMSEP (25.7 g l^{-1}), the control of total sugar concentrations during the fed-batch phase was poor. Partially, sugar levels exceeded the target setpoint of 45 g l^{-1} by 25 g l^{-1}. The model also revealed a significant negative bias with an average actual total sugar concentration of 65 g l^{-1}.

In contrast, the control performance of the final calibration model (Table 2) was visibly improved (Figure 6). Total sugar levels were maintained within 5 g l^{-1} of the target value of 45 g l^{-1} during the fed-batch phase and the average error of prediction was -0.45 g l^{-1} resulting in a smooth control of target total sugar concentrations. From an initial fructose : glucose ratio of 1:1, concentrations of these hexoses diverged within the first 50 h of incubations and then levelled off at 30 and 15 g l^{-1} for fructose and glucose, respectively, resulting in a 2:1 ratio.

Discussion

The application of a novel fed-batch technique was shown to improve viability of *S. cerevisiae* during alcoholic fermentation of a white grape must, and to greatly reduce the formation of the osmotic stress related by-product acetic acid (Frohman and Mira de Orduña, 2013). Sugar concentrations were held constant during these fermentations by continuously feeding grape must at variable rates. Practical implementation of this method thus requires process automation involving a relatively high measurement frequency and continuous feeding rate adjustment. The current work details the creation and validation of a FT-NIR spectroscopy-based calibration model for total sugars and its integration with a process control system to automate the realization of constant substrate-level fed-batch fermentations. In addition, calibration models for fructose, glucose and the main fermentation product ethanol were also established to promote process monitoring.

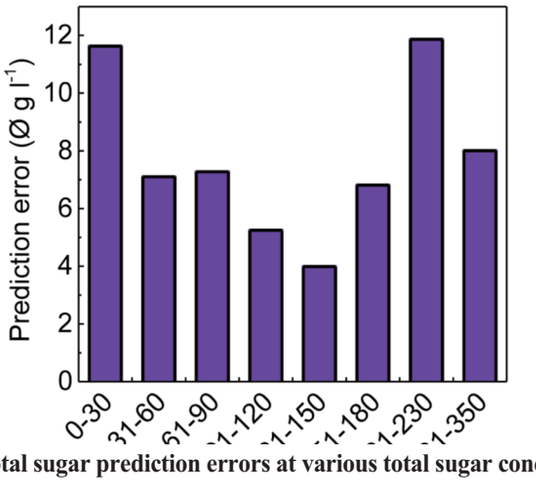


Figure 4. Average absolute total sugar prediction errors at various total sugar concentration ranges (final model)

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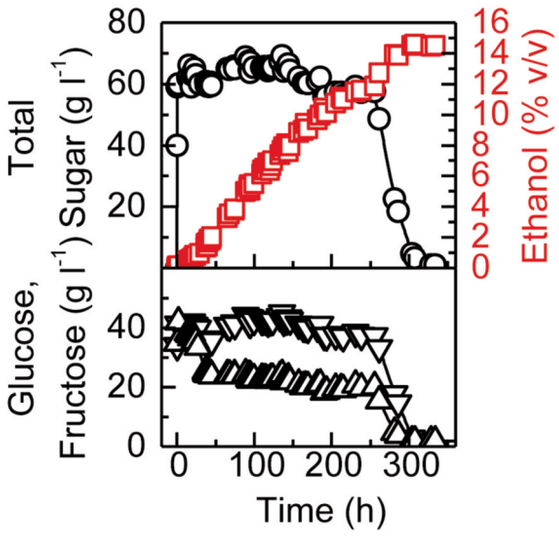


Figure 5. Time course of total sugar (-), ethanol («), glucose (8) and fructose (C) concentrations quantified by HPLC for the fed-batch validation fermentation automatically controlled using the initial total sugar model (N = 33). Target total sugar setpoint, 45 g l^{-1} ; inoculation rate, 40 g hl^{-1}.

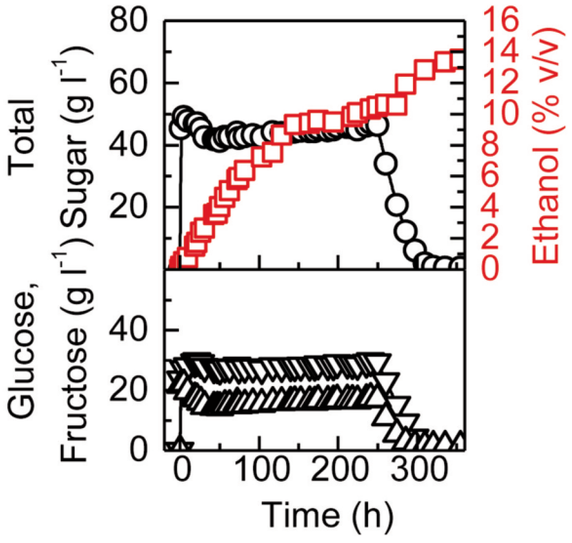


Figure 6. Time course of total sugar (-), ethanol («), glucose (8) and fructose (C) concentrations quantified by HPLC for the fed-batch validation fermentation automatically controlled using the final total sugar model (N = 242). Target total sugar setpoint, 45 g l^{-1} ; inoculation rate, 40 g hl^{-1}.

NIR spectroscopy-based calibrations with suitable prediction errors have previously been developed for the quantification of various grape and wine constituents, including sugars (Manley *et al.*, 2001; Urbano-Cuadrado *et al.*, 2004; Niu *et al.*, 2008; Di Egidio *et al.*, 2010). However, these models were developed using filtered, centrifuged, or chemically clarified samples and consequently exhibited low turbidity levels. The creation of accurate calibration models is feasible under such conditions. A pre-experiment by the authors found that it was possible to create NIRS-based models for the quantification of total sugars, glucose, fructose and ethanol in fermenting wines with R^2 values > 0.999, RMSECV values < 1 (<0.15 for ethanol), and RPD values > 40 when 50 sterile-filtered fermentation samples were utilized (data not shown). Unfortunately, such models can only be used for offline analysis.

In contrast, the high turbidity of active fermentations renders acquisition of high quality spectra and generation of accurate and robust models challenging. In the current work, 33- and 55-fold differences in yeast nutrient and active dry yeast concentrations, respectively, were used among the training challenges to build differences in sample turbidity into the developed models. Simultaneously, the probe used for spectral acquisition as well as the acquisition settings were optimized. The transflectance-type NIR spectroscopy probe used simultaneously measures transmitted and back-scattered radiation (Osborne and Fearn, 1986; VonBargen, 1996; Ozaki *et al.*, 2007). Compared to test spectra recorded using a transmission probe, the transflectance probe spectra demonstrated a 1-log decrease in baseline absorbance levels and a significant reduction in background noise. To further improve the signal to noise ratio, sample scan time and spectral resolution were also optimized. Sample signal and pre-amplification gains were maximized while preventing detector saturation (Cervera *et al.*, 2009).

The fermentation vessel design and measurement arrangement were also improved for spectral collection. Since biomass sedimentation and adhesion of CO_2 bubbles on the transflectance probe head resulted in unrepresentative spectra, a recirculation loop was thus added in the final design of the system reported, and the transflectance probe installed directly into the flow path. The fast-moving fermentation liquid thus continuously flushed the probe head and prevented build-up. The inclusion of a recirculation loop also ensured rapid and thorough mixing, prevented temperature gradients and minimized the delay between feed addition and reaching fermentation homogeneity.

The initially generated total sugar model was not sufficiently robust to describe matrix variation during fed-batch fermentations. In contrast, the final models

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consisting of approximately 240 standards and including spectra from several automated and continuous fed-batch fermentations provided acceptable models for all four parameters. Calibration model RPD values ranged from 3.83 (glucose) to 9.14 (ethanol). The final total sugar model had an intermediate RPD value of 6.01. All of these values are above the accepted screening limit (3), with 6.01 (total sugars) being above the limit for quality control and 9.14 (ethanol) being beyond the cut-off for all analytical tasks (Williams, 2004 ; Conzen, 2006). While the number of variables utilized for the three sugar models is comparatively high, it is low in comparison to other developed sugar NIRS models (Chung *et al.*, 1996 ; Arnold *et al.*, 2003 ; Urbano-Cuadrado *et al.*, 2004 ; Petersen *et al.*, 2010) which may exhibit an increased risk of over-fitting due to the inclusion of noise features in the model. With 242 training standards utilized in the final total sugar model, the RMSEP had not asymptotically approached a lower limit (Figure 3), thereby suggesting the potential for further improvement via the inclusion of additional standards. However, the final model reported here already sufficed the requirements for maintaining total sugar concentrations constant during fed-batch fermentation under practical high turbidity conditions. The accuracy of the calibration model was further demonstrated by carrying out the validation fed-batch fermentation with a thermovinified and not previously filtered red grape must, whereas the calibration models had been developed using white wine samples.

By integrating in-line sugar analysis with process control, the system presented herein enables the automation of constant substrate-level fed-batch vinifications. While NIRS is widely used as an analytical tool in various bioprocesses, its use in combination with automation modules to control and direct fermentations is still limited (Macaloney *et al.*, 1996 ; González-Vara *et al.*, 2000 ; Berraud, 2000 ; Tosi *et al.*, 2003 ; Navrátil *et al.*, 2005). To the best of the authors' knowledge, this is the first time NIRS has been used to automatically control a fermentation process in oenology.

A chemostat is a continuous microbiological cultivation method at a constant-volume where the rate of inflowing (and hence outflowing) growth medium is adjusted (Bull, 2010). In analogy, substratostat is proposed as designation for the current type of cultivation since the concentration of the main substrate, the carbon and energy source, is held adjusted during fermentations. In a chemostat, the manually adjusted experimental variable « flow rate » allows to indirectly define the microbial growth rate μ within certain limits (Bull, 2010). In the current substratostat, the flow rate is automatically adjusted based on the metabolic activity of the production organism. It is thus a fed-batch with direct and automatic feedback control according to the classification of Yamanè and Shimizu (1984).

Conclusions

FT-NIRS calibration models for the continuous in-line determination of total sugars, glucose, fructose and ethanol in fully turbid and actively fermenting grape musts were established. NIRS quantification of total sugars was integrated with process automation technology to create a fully automated system for conducting variable-feed fed-batch vinifications at constant substrate (sugar) concentrations. By maintaining low sugar concentrations (<100 g l^{-1}) during the fermentation of musts this innovative substratostatic system allows for the prevention of the hyperosmotic stress response in *S. cerevisiae*. With suitable prediction models, this system may also find application in other bioprocesses benefitting from improved control of substrate concentrations.

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