Innovative Romanian Food Biotechnology *Vol. 12, Issue of March, 2013*

Received January 30, 2013 / Accepted March 22, 2013

RESEARCH ARTICLE

VITALTITRATION USED FOR PREDICTING FERMENTATION PERFORMANCE OF ETHANOL STRESSED BREWING YEAST

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Abstract

Obtaining different batches of beer with the same characteristics each time is a continuous challenge for brewers worldwide. Besides the complexity and natural variation of ingredients´ composition, reusing the same batch of yeast up to 7-10 times makes the brewing process even more difficult to control. The pitching brewing yeast should have a high metabolic activity in order to meet the expected fermentation performance. Aiming to determine the fermentative performance of brewing yeasts stored under ethanol we have used a vitality assay. The physiological state of yeast cells stored for 72h in the presence of ethanol was evaluated in terms of vitality, measured as CO_2 production and H^+ extrusion. Two widely used European industrial brewing yeast strains, *Saccharomyces lager* W 34/70 and *Saccharomyces ale* W 210, were stored in conditions similar to the ones used for industrial brewing yeast cream. Our results show that the *Saccharomyces ale* W210 strain can be stored for larger storage periods than W34/70, but the ethanol stress should be of maximum 7.5% (v/v) ethanol to have an acceptable loss of cells' vitality during storage. These informations can be valuable for fermentation performance prediction of the two tested industrial brewing yeast starter cultures, as well as for a correct dimensioning of the yeast inoculum.

Keywords: yeast vitality, physiological state, beer, storage, yeast quality

Introduction

A challenging characteristic of the brewing process is serial repitching (Boulton and Quain, 2006; Lodolo *et al.*, 2008), which refers to cropping, refrigeration storage and recycling of yeast biomass on completion of fermentation. If the quality characteristics of stored yeast are suitable (contamination free, good viability, satisfactory organoleptic characteristics, optimal cell density), the same inoculum of yeast is reused for 10 - 12 fermentation batches (Boekhout and Robert, 2003; Guido *et al.*, 2004).

When analyzing a yeast population to be used as inoculum for a new fermentation batch it is expected that part of the cells will not have all the characteristics of viable cells. For example, it is possible that they cannot multiply but are able to participate in the fermentation process by metabolizing the nutritive substances from the wort

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and contribute to the production of beer aroma compounds.

The use of yeasts in brewing industry is associated with their exposure to severe environmental changes throughout the propagation and fermentation process, as well as during yeast storage process (van Voorst *et al.*, 2006; Gibson *et al.*, 2007). One of the type of stress yeast encounters during the brewing process is ethanol stress and although brewing yeasts are able to tolerate high levels of ethanol, its accumulation during the fermentation process and persistence during storage is frequently associated to sub-optimal yeast fermentation performance (Lentini *et al.*, 2003). Ethanol primarily targets membranes, increasing their fluidity and permeability, ultimately affecting the transport system of essential compounds such as aminoacids and glucose. Furthermore, ethanol accumulation compromises a range of cellular functions leading to the reduction of cell metabolic rate, growth and viability (Alexandre *et al.*, 2001; Lentini *et al.*, 2003; Garay- Arroyo *et al.*, 2004; Gibson *et al.*, 2007; Pizzaro *et al.* 2007).

The effects of a stressor upon a microorganism culture are usually quantified by determining its viability. By using viability tests, discriminating between dead and live cells, in brewing industry a correction factor can be established to assure the same concentration of viable cells for all fermentation batches. Unfortunately, viability tests are not able to predict the ratio between the total number of cells and the above mentioned type of cells (viable or dead). Under these circumstances, vitality assays are a good complementary option, being able to indicate the physiological state of the brewing yeast cells. High vitality results in a fast fermentation with minimal undesired by-products, while low vitality triggers sluggish or poorly attenuating fermentation. During the last two decades, several types of tests have been developed for vitality assessment (for review see Lentini *et al.*, 1993; Imai, 1999; Heggart *et al.*, 2000; White *et al.*, 2003; Sigler *et al.*, 2009), offering valuable information for fermentative performance forecasting. Among the available vitality tests some are based on cellular composition (level of glycogen or trehalose assay, monitoring sterol level, intracellular ATP concentration assay by bioluminiscence, concentration of NAD/NADH by fluorescence spectroscopy) (Boulton and Quain, 2006), others are based on evaluation of cellular activity (fermentation tests during which specific velocity of oxygen consumption is analyzed, speed of carbon dioxide or ethanol production) (Boulton and Quain, 2006), acidification power tests – based on spontaneous acidification or as a response to glucose supplementation (Morata *et al.*, 2003; Kuřec *et al.*, 2009) or using fluorimetric vitality tests (Heggart *et al.*, 2000).

This study aimed to evaluate vitality of two starter cultures of brewing yeast cells stored for 72 h under ethanol stress conditions, at refrigeration temperature (6 ˚C) and above the upper limit of refrigeration temperatures, 12 ˚C respectively. Vitality assessment was performed by vitaltitration method (Rodrigues *et al.*, 2004; Magalhães *et al.*, 2011).The results are expected to be useful for evaluating the ethanol tolerance of the two industrial brewing strains by estimating the physiological changes caused by their storage under different stress conditions. The practical importance of the vitality results resides in the ability to correlate directly with the yeast metabolic competence, enabling a clear differentiation between yeast with variable fermentation capacities.

Materials and methods

Yeast strains and growth conditions

Two brewing yeast strains were used, the bottom-fermenting *Saccharomyces ale* W 34/70 strain and the top fermenting *Saccharomyces lager* W 210 strain, both acquired from Weihenstephan Hefebank, Germany. For yeast propagation ~10^6 cells/mL were inoculated on liquid YPD medium (yeast extract 10 g, bactopeptone 20 g, glucose 20 g, distilled water to 1000mL) and cultivated for 48 h at 27 ˚C with continuous agitation of 200 rpm. The resulting yeast biomass was centrifuged at 5000 rpm for 10 min at 4˚C and further used for storage under ethanol stress. The propagations were independently replicated three times for each yeast strain.

Yeast cream storage under ethanol stress conditions

To evaluate the influence of ethanol stress during brewing yeast storage, yeast cream (aprox. 10^{11} cells/mL) was suspended in 0.1M acetate buffer pH 4.2 and kept for 72 h under three different ethanol stress conditions (final ethanol concentration 5%, 7.5%, 9% v/v) and two temperatures, of 6 ºC and 12 ºC respectively. As control a sample with the same yeast cell concentration was used and sterile distilled water was added instead of ethanol. One control sample was made for each of the tested temperatures (6 ºC and 12 ºC). During the 72 h of storage samples were collected daily for yeast cell vitality assay.

Vitality assay for ethanol stressed brewing yeast

Sampled biomass was first centrifuged at 5000rpm for 10min, at 4˚C, and then washed four times with refrigerated distilled water followed by cold centrifugation at 5000rpm for 10min. The resulting yeast biomass was used for vitality assay performed with vitaltitration method (Rodrigues *et al.*, 2004) with minor adaptations. Evaluation of the physiological state of yeast cells stored for 72 h in the presence of ethanol was based on the measurement of CO_2 production and H^+ extrusion, which reflects the yeast metabolic activity.

The physiological explanation of the process is that the pH variation is closely related to the activity of the membrane-bound H^+- ATPase. For maintaining the intracellular pH as well as for preservation of the electrochemical proton gradient across the plasma membrane, cell has to discard protons. This gradient is responsible for active transport of nutrients into the cell which is essential for cell viability and as a result is correlated to the metabolic activity during fermentation.

In practice, yeast biomass is resuspended in NaCl 0.9% at 27 ºC, its pH is raised to 10.0 with 0.1 M NaOH and the yeast suspension is incubated with constant stirring while the pH drop in time is monitored until it reaches 6.5. A yeast metabolic index (YMI) is calculated, using equation (1):

$$YMI = \frac{[NaOH]\cdot V_{NaOH}\cdot 10^6}{\tau_{pH\ drop}}, \mu mol\ NaOH/min \quad (1)$$

The drop of pH is primarily a consequence of CO_2 production and H^+ extrusion, both of which are indicators of the cell's metabolic activity. Moreover, since during the assay no exogenous energy source is added, the CO_2 production and H^+ elimination are dependent on intracellular energy reserves, also related to yeast vitality. The vitaltitration method may therefore reflect, in an indirect manner, the level of the intracellular energy reserves. In short, the vitaltitration method relates the time that the yeast takes to acidify the solution from pH 10.0 to pH 6.5 with its vitality. The more vital the cells are the less time they take to acidify the solution and the higher is the YMI value (Rodrigues *et al.*, 2004).

Results and discussion

Yeast storage parameters were chosen to mimic the industrial brewing conditions at laboratory scale. The pH of the storage suspension (0.1M acetate buffer pH 4.2) was chosen to fit both types of brewing yeast fermented worts (lagers: 4.2 - 4.75 and ales: pH 3.8 - 4.2) (Bamforth, 2001; Hornsey, 2003).

Between two fermentation batches yeast are usual stored at refrigeration temperature, between 2 and 6 ºC. Research studies (Boulton and Quain, 2006) indicate that when storing 90 kg of yeast cream at 1-2 ºC, the temperature in the center of the vessel reaches 10-12 ºC due to the metabolic exergonic metabolic reactions. Considering these results, 6ºC and 12 ºC were chosen as tested temperatures for storage, representing refrigeration temperature and a temperature above the recommended refrigeration temperature.

Brewing yeast cream is usually stored at refrigeration temperatures for maximum 72h (Boulton and Quain, 2006), so tests were performed on yeast biomass stored under different ethanol stress conditions for the abovementioned period.

Vitality of Saccharomyces lager W 34/70 brewing yeast stored under ethanol stress

Brewing fermentation performance depends not only of yeast viability, which is commonly tested

in breweries, but also of yeast vitality, a less common used yeast quality parameter. Storage under ethanol affects yeast vitality to a greater extent compared to other types of stress, such as thermal or osmotic stress for example (Weigert, 2009).

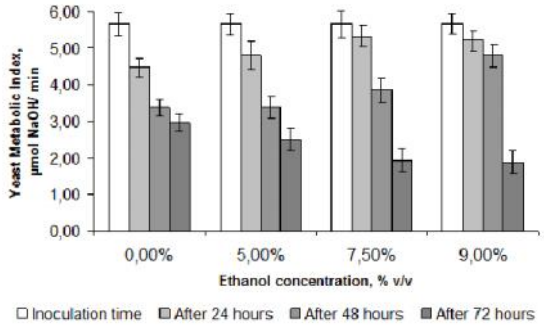

Figure 1. *Metabolic index variation for Saccharomyces lager W 34/70 strain stored at 6ºC for 72h under 0.1M acetate buffer, pH 4.2. Results are shown as average of three independent experiments and error bars are representative of standard deviation*

The vitality results obtained for *Saccharomyces* lager W 34/70strain stored at 6 ºC (Figure 1) show an apparently contradictory result after the first 24 h of storage: the higher is the ethanol concentration the more vital are the cells (e.g. vitality for 7.5% ethanol sample or 9% ethanol sample is increased, with 11.25% and 8.54% respectively, compared to vitality of yeast cells stored under 5% ethanol).

The explanation resides in the adaptation process of the survivor yeast cells to ethanol stress, which acts as an enhancer of yeast vitality. The fact is in accordance with reality, since the vitality test only refers to the live yeasts' metabolic activity and does not include reference on the number of living cells. The following 24 h of storage at 6 ºC trigger a 30% decrease in vitality for both blank and 5% ethanol sample. This is an indication that yeast can cope well with 5% ethanol for 48 h storage at 6 ºC. Further on YMI increases with the ethanol concentration: 14% more for the 7.5% ethanol sample and 42% more for the 9% ethanol compared to control sample after 48 h. The metabolic indexes obtained for the *Saccharomyces* W34/70 strain stored for 72 h at 6 ºC showed that the reduction in vitality was related to the increase in ethanol concentration, e.g. when ethanol concentration was high the cells became less vital. These results were in agreement with those observed in industry, although they refer to freshly cropped yeasts in exponential growth phase (Rodrigues *et al*., 2004). Other vitaltitration results on vitality dynamic during storage under physiological conditions performed with a different lager yeast strain, NCYC1324, indicate a nearly 55% loss of vitality after three days of storage at 12 ºC and about 36% reduction when lager yeast slurry is stored at 4 ºC.

The results from the metabolic index analysis indicate the following hypothesis: for *Saccharomyces* lager W34/ 70 strain stored at 6 ºC, 7.5% (v/v) ethanol seems to be an adaptability threshold, since there was no significant increase in YMI above this value that could indicate an increased adaptation of the response to stress. Adaptability of yeast cells that succeed to survive under ethanol stress of maximum 7.5% (v/v) could also trigger the mechanisms of response to increased stress. As consequence, vitality of yeast stored for 48 h under 9% (v/v) ethanol is higher compared to the one for the stored reference sample or with 5% (v/v) ethanol.

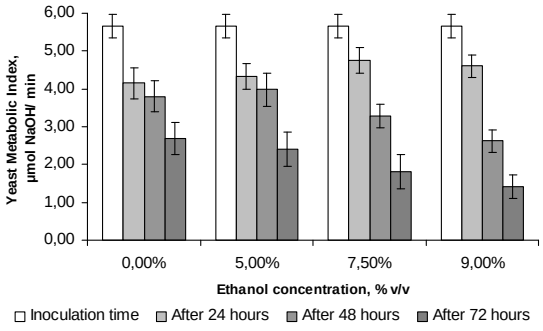

Figure 2. *Metabolic index variation for Saccharomyces lager W 34/ 70strain stored at 12ºC for 72h under 0.1M acetate buffer, pH 4.2. Results are shown as average of three independent experiments and error bars are representative of standard deviation*

Analyzing the results for metabolic index of W 34/70 yeast stored under ethanol stress at 12 ºC (figure 2) one can observe that after the first 24 h of storage the surviving stressed lager yeast exhibit an increased metabolic activity: for 5% (v/v) ethanol there is an improvement of 7% compared to the blank sample, of 19% for the 7.5% (v/v)

ethanol sample and of 16% respectively for the 9% (v/v) ethanol sample. These results support again the hypothesis according to which 7.5% (v/v) ethanol is an adaptation threshold value for the lager yeast stored under temperatures exceeding the refrigeration interval, mentioned before.

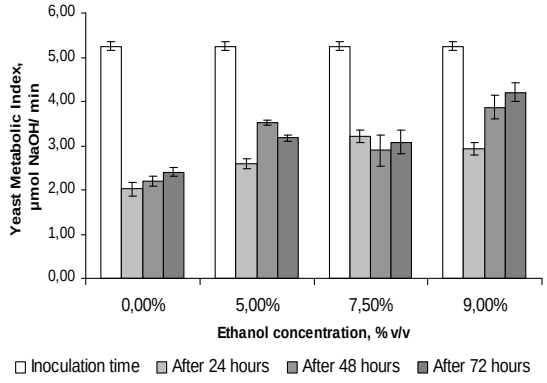

Figure 3. *Metabolic index variation for Saccharomyces ale W 210 strain stored at 6ºC for 72h under 0.1M acetate buffer, pH 4.2. Graph represents average of three independent experiments and error bars are representative of standard deviation*

The data show that when *Saccharomyces* W 34/ 70 cells are stored at 12 ºC there is a dependence of vitality with the intensity of ethanol stress that generates a response: compared to the control sample the YMI is increased with 4% for sample with 5% (v/v) ethanol, with 14% for the 7.5% (v/v) ethanol and with 10.6% for the 9% (v/v) ethanol sample. The storage prolonged more than 24 hours triggers a reduction of the physiological activity of yeast from all samples: after 48 hours of storage the YMI is 9% lower for the blank sample, with 8% less for the 5% (v/v) ethanol, 30% less for the 7.5% (v/v) ethanol sample and 57% less for the 9% ethanol (v/v) respectively, all compared to the values obtained after 24 h of storage. The 72 hours of storage reduces even more the metabolic index: 30% less for the no-ethanol sample, 40% less for the 5% (v/v) ethanol, 45% less for the 7.5% (v/v) ethanol and 54% less for the 9% (v/v) ethanol sample, all compared to the previous values, obtained after 48 hours of storage.

Comparing the results of metabolic index variation for *Saccharomyces* W 34/ 70 strain stored under ethanol stress at different temperatures it can be seen that higher temperature (12ºC compared to 6 ºC) triggers a more abrupt lowering of the metabolic index under ethanol stress conditions.

When analyzing vitalities of yeast stored at 6ºC and 12ºC temperatures there is no indication of a notable difference between vitality of yeast stored under physiological conditions and the one with 5% (v/v) ethanol. The situation changes when 7.5% (v/v) ethanol is used: *Saccharomyces* lager yeast W 34/ 70 has a decrease in viability of 32% after two days of storage at 6 ºC and of 42% when the storage temperature rises at 12 ºC. When the couple temperature- ethanol stress is at higher values (9% ethanol, 12 ºC), vitality drops with 54%, compared to only 15% when storage temperature is 6 ºC, for two days.

When the storage temperature is above the upper limit of refrigeration temperature, the yeast metabolic index degradation is much more rapid in the case of the *Saccharomyces* lager W 34/70 strain, which is probably caused by the cumulative effect of thermal and ethanol stress.

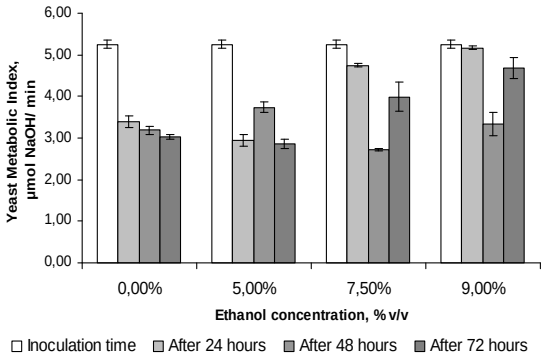

Figure 4. *Metabolic index variation for Saccharomyces ale yeast W 210 strain stored for 72h at 12ºC, under acetate buffer 0.1M, pH 4.2. Graph represents average of three independent experiments and error bars are representative of standard deviation*

Vitality of Saccharomyces ale W 210 brewing strain stored under ethanol stress

Same tests were performed for *Saccharomyces* ale W 210 yeast strain and variation of metabolic indexes is graphed in Figure 3 (storage at 6 ºC) and Figure 4 (storage at 12 ºC). A significant loss of vitality is observed after one day of storage, even for the blank sample: 61% for the ale yeast stored at 6 ºC and 36% for samples stored at 12 ºC. This

could be due to ale yeast characteristic of optimum fermentation temperature, ranging between 14 ºC and 25ºC (Pugh and Ryder, 2003).

The ale yeast sample treated with 5% (v/v) ethanol and stored at 6ºC shows a 27% improvement of metabolic activity after 48 h of treatment, while for the other two samples with 7.5% (v/v) and 9% (v/v) ethanol respectively the melioration appears only after 72 h of ethanol treatment.

When analyzing the variation of metabolic index for *Saccharomyces* ale yeast W 210 strain stored under ethanol stress at 12 ºC (Figure 4) it can be observed that vitality is reduced much slower than in the case of storage for 24 h at 6 ºC, correlating this information with the ale yeast preference for higher temperatures compared to lager yeasts. Yeast stored under 5% (v/v) ethanol have an improved vitality after 48 hours of storage, while for the samples stored with 7.5% and 9% (v/v) ethanol the improvement appears after 72 h of storage. This behavior could be an indication of adaptability for the yeast surviving the 72 h of ethanol stress.

The metabolic indexes for the ale strain W210 showed a significant loss of vitality after 24 h of storage at 6 ºC for all samples tested (Figure 3). At this temperature ethanol improves cell vitality after 48h of storage when compared to the blank sample: 28% improvement for the 5% (v/v) ethanol, 59% higher for the 7.5% (v/v) ethanol sample and with 45% bigger for the 9% (v/v) ethanol. The reduced vitality was partially alleviated when cells were stored at 12ºC (Figure 4), consistent with the fact that *ale* yeasts are known to be more sensitive to low temperatures than *lager* yeasts (Verbelen, 2009). Under these conditions, ethanol concentrations higher than 7.5% (v/v) clearly increase cell vitality after 24 h of storage. Therefore, *Saccharomyces* ale W210 strain exhibited higher vitality in the presence of high ethanol concentrations and elevated storage temperature. Unlike to what happens with *Saccharomyces* lager W34/70 strain, the increased vitality is maintained even upon 72h storage (compare Figure 2 and Figure 4) of W 210: 32.5% increase for the 5% (v/v) ethanol, 28% higher for the 7.5% (v/v) ethanol and 75% higher for the 9% (v/v) ethanol.

A comparison of the viability and vitality leads to the conclusion that *Saccharomyces* lager W34/70 and *Saccharomyces* ale W210 cells stored at 6 ºC for 72 h in the presence of ethanol concentrations up to 7.5% (v/v) ethanol present low vitality although they are considered to be in good condition to proceed with their repitching (compare Figure 1 and Figure 2). Thus, to predict with good confidence the fermentative performance of a brewing yeast inoculum it is necessary to take into consideration many aspects of yeast biology, such as the combination of optimal parameters for viability as well as for vitality maintenance.

Conclusions

Prediction of fermentative performances for the brewing yeast inoculum is always a difficult task given the fact that yeast behavior is dependent of numerous metabolic events. As such, numerous tests for evaluation of fermentative capacity have been developed. This study used vitaltitration method for estimating vitality of brewing yeast stressed by ethanol doubled by temperature through storage at temperatures higher than the recommended ones(i.e. refrigeration temperatures).

The negative synergic effect of ethanol and temperature as stress factors upon vitality for yeast stored at temperatures higher than the recommended ones, namely the refrigeration ones, is proved by the performed vitality assays. In practice, the reduction of vitality is directly linked to fermentative performances of the yeast inoculum, leading to variable beer quality between different batches.

Our study can be valuable for brewing industry by indicating the maximum storage periods of yeast cream under ethanol stress combined with higher temperatures: i.e. for an acceptable loss of vitality during storage, *Saccharomyces* ale strain W210 can be stored for larger periods than *Saccharomyces* lager W34/70 strain, under an ethanol stress of maximum 7.5% (v/v), for maximum 72 h.

In extreme situation, when ethanol/ temperature stressed inoculum that should be discarded has to

be used in a brewing fermentation batch, our vitaltitration results can help resizing the inoculum so that the poor quality of yeast cells will be improved by a higher quantity of inoculum.

The results of yeast vitality stored without ethanol stress are appropriate to accurately forecast the fermentation performance of the tested yeast strains under custom conditions.

Our data show that vitaltitration is a rapid and easy assay to perform, requiring typical brewery laboratory equipment and regular reagents that can be easily employed in the brewing industry for improving brewing yeast management.

Acknowledgement

The authors wish to thank *Bioaliment* Research Platform from "Dunarea de Jos" University in Galati- Romania, Food Science and Engineering Faculty, for the research infrastructure provided for this study.

References

Alexandre H., Ansanay-Galeote V., Dequin S., Blondin B. (2001) Global gene expression during short-term ethanol stress in Saccharomyces cerevisiae, FEBS Letters, 498, 98-103.

Bamforth C.W. (2001) pH in Brewing. An Overview, *MBAA TQ*, 38, 1-9.

Boekhout T., Robert V. (2003) Yeast in Food. Behr, Hamburg, Germany.

Boulton C., Quain D. (2006) Yeast Management. Yeast vitality tests. In Boulton C. and Quain D. (eds.): *Brewing Yeast and Fermentation*, 501-508, Blackwell Publishing House.

Garay- Arroyo A., Covarrubias A. A., Clark I., Nino I., Gosset G., Martinez A. (2004) Response to different environmental stress conditions of industrial and laboratory *Saccharomyces cerevisiae* strains, *Appl. Microbiol. Biotechnol.*, 63, 734-741.

Gibson B. R., Lawrence S. J., Leclaire J. P., Powell C. D., Smart K. A. (2007) Yeast response to stresses associated with industrial brewery handling, *FEMS Microbiological Review*, 31, 535-569.

Guido L. F., Rodrigues P. G., Rodrigues J. A., Goncalves C. R., Barros A. A. (2004) The impact of the physiological condition of the pitching yeast on beer flavour stability: an industrial approach, *Food Chem*., 87, 187-193.

Rodrigues P. D., Barros A. A., Rodrigues J. A., Ferreira A. A., Goncalves C. (2004) Vitaltitration: A New Method for assessment of Yeast Vitality. *MBAA TQ*, 41, 277-281.

Heggart H. M., Margaritis A., Stewart R.J., Pilkington H., Sobczak J, Russell I. (2000) Measurement of brewing yeast viability and vitality: a review of methods. *Tech. Q. Master Brew. Assoc. Am*., 37, 409-430.

Hornsey I. (2003) A History of Beer and Brewing, *Royal Society of Chemistry Paperbacks*, Cambridge, UK.

Imai T. (1999) The assessment of yeast vitality - the past and the future. *Brew. Guardian*, 128, 20-27.

Kuřec M., Kuncova G., Branyik T. (2009) Yeast vitality determination based on intracellular NAD(P)H fluorescence measurement during aerobic–anaerobic transition. *Folia Microbiol*., 54, 25-29.

Lentini A. (1993) A review of the various methods available for monitoring the physiological status of yeast: yeast viability and vitality. *Ferment*, 6, 321-327.

Lentini A., Jones R. D., Wheatcroft R. (2003) The impact of ethanol stress on yeast physiology. In Smart K (ed.): *Brewing Yeast Fermentation Performance*, Second Edition, 25-37, Blackwell Publishing.

Lodolo E. J., Kick J. L. F., Axcell B. C., Brooks M. (2008) The yeast Saccharomyces cerevisiae– the main character in beer brewing, *FEMS Yeast Research*, 8, 1018-1036.

Magalhães P.J., Carvalho A. B., Goncalves L. M., Pacheco J. G., Guido L. F., Branyik T., Rodrigues P. G., Kuncova G., Dostalek P., Barros A. A. (2011) The Impact of Xanthohumol on a Brewing Yeast's Viability, Vitality and Metabolite Formation, *J. Inst. Brew*. 117, 368–376.

Morata A., Gomez- Cordoves M. C., Suberviola J., Bartolome B., Colomo B., Suarez J. A. (2003) Adsorption of anthocyanins by yeast cell walls during the fermentation of red wines. *J. Agric. Food Chem*., 51, 4084-4088.

Pizzaro F., Vargas F.A., Agosin E. (2007) A System Biology Perspective of Wine Fermentation, *Yeast*, 24, 977-991.

Pugh T., Ryder D. (2003) Art of Brewing and Biology of lager yeast. In: Newsletter of *Saccharomyces* Genome Database, October 2003. Available from Internet: http://www.yeastgenome.org/community/ArtOfBrewing.htm

Sigler K., Matoulkova D., Dienstbier M., Gabriel P. (2009) Net effect of wort osmotic pressure on fermentation course, yeast vitality, beer flavor, and haze, *Appl. Microbiol. Biotechnol*., 82, 1027-1035.

vanVoorst F., Houghton- Larsen J., Jonson L., Kielland-Brandt M. C., Brandt A. (2006) Genome- wide identification of genes required for growth of *Saccharomyces cerevisiae* under ethanol stress, *Yeast*, 23, 351-359.

Verbelen P.J., Dekoninck T. M., Saerens S. M., Van Mulders S. E., Thevelein J. M., Delvaux F. R. (2009) Impact of pitching rate on yeast fermentation performance and beer flavour, *Appl Microbiol Biotechnol*, 82, 155-167.

Weigert C., Steffler F., Kurz T., Shellhammer T. H., Methner F.J. (2009) Application of a short intracellular pH method to flow cytometry for determining *Saccharomyces cerevisiae* vitality. *Applied and Environmental Microbiology*, 75, 5615–5620.

White L.R., Richardson K.E., Schiewe A. J., White C. E. (2003) Comparison of yeast viability/vitality methods and their relationship to fermentation performance. In: Smart K (ed.): *Brewing Yeast Fermentation Performance*, Second Edition, 138-147, Blackwell Publishing.

Quain D. E. (1988) Studies on yeast physiology-impact on fermentation performance and product quality, J. Inst. Brew., 95, 315-323.