F. Thiele and W. Back

Measurement of yeast vitality using a modified version of the intracellular pH measurement (ICP)

The measurement of the intracellular pH using the ICP-Method has proofed to be sensitive and reliable indicator of the physiological condition of yeast. This method has the drawbacks that uncommon equipment (spectrofluorophotometer) is necessary and that even under optimal condition the analysis takes 3.5 hours and is very labour intensive. Especially this time consuming characteristic makes this analysis extremely unattractive for a routine use in a brewery laboratory. To overcome this drawback an alternative sample preparation was developed which shortens the time for this method to approximately half an hour. The results of this Short ICP method (S-ICP) demonstrate a very good correlation with the original method. The requirement of a spectrofluorophotometer still persists.

Descriptors: Yeast Vitality, intracellular pH value.

1 Introduction

The vitality of the pitching yeast is a crucial factor for the fermentation time and has a great influence on the quality of the resulting beer. To evaluate the physiological condition of the living yeast cells several methods have been developed. Most of these methods are based on the content of a single cell compound or a single yeast activity. It is doubtful that a single factor is able to define the overall physiological condition of the cells and most methods have been found to have certain limitations and drawbacks. Imai et al. developed a method to measure the intracellular pH as an indicator for the yeast vitality (1). In different studies this method has demonstrated its practical value as a sensitive and reliable indicator for the physiological condition of yeast (1,2,3). According to Imai the importance of the intracellular pH for the yeast physiology is based on several factors. For example the plasma membrane ATPase regulates the intracellular pH, which is essential for yeast growth (4). The transmembrane H^+ gradient as a result of the plasma membrane ATPase activity is a driving force for the uptake of nutrients (5,6,7,8). Furthermore the intracellular pH regulates key enzymes in glycolysis and gluconeogenesis by the regulation of the c-AMP (9,10,11). The c-AMP plays an important role in the regulation of enzymes like fructose-1,6-bisphosphatase (12,13) and 6-phosphofructo-2-kinase (14,15,16).

Several techniques have been developed to determine the intracellular pH of yeast including distribution of weak acids (17,18,19), the ^{31}P nuclear magnetic resonance technique (20,21,22,23,24,25), and the application of pH-dependent fluorescence probes (26,27,28,29).

Depending on the used conditions like the extra cellular pH and the method incorporated different intracellular pHs where found. Different explanations can be found for these deviations (28).

The method developed by Imai et al. is based on the pH-dependent fluorescent dye Carboxyfluorecein Diacetate (CF-DA). As mentioned before one important factor for the intracellular pH is the employed extra cellular pH. Imai et al. used their conventional method to determine the intracellular pH of resting cells a pH of 6.2 as the buffer pH to maintain a certain pH level during the sample preparation and the incubation with the pH dependent fluorescent dye (30). In order to measure yeast vitality instead of determine just the actual intracellular pH of the yeast Imai et al. changed the buffer pH to 3 because the intracellular pH of active cells does not decrease even if the extra cellular pH is low. In contrast the intracellular pH of a less active cell does decrease at a low extra cellular pH (1).

During the sample preparation for the ICP-method the yeast is kept on ice to maintain low temperatures. This is necessary because most fluorescent dyes suitable for the intracellular pH measurement are accumulated in the vacuoles and / or actively extruded to the extracellular environment. This happens especially in energized cells. This can lead to erroneous result since the vacuole has a different pH as the cytosol and extracellular fluorescent dye result in high background levels of fluorescence (28). The low temperatures and exclusion of external energy sources like glucose through several washing steps prevent the mentioned problems. But this leads as well to long sample preparation times. Under optimal condition the analysis takes approximately 3.5 hours and one operator is busy with the sample preparation most of these 3.5 hours. This makes the method not suitable for routine analysis. This work deals with the development of modified sample preparation to reduce the time significantly by combining the ICP method with the experience of other authors using the same fluorescent dye.

2 Material and methods

2.1 Yeast

Two top-fermenting yeast strains W68 and W175 and two bottom-fermenting yeast strains W34 and Hebru in different physiological conditions were used.

2.2 ICP Method

The yeast slurry was filtered with a wire netting (400 mesh) to remove precipitates. A 2-mL yeast pellet was collected by centrifugation (2000 x g for 3 min at 2 °C). All centrifugations were carried out under these conditions, unless otherwise mentioned. The pellet was washed twice with cold 8 mL of a 50 m*M* citric disodium hydrogen phosphate buffer (pH 3.0, containing 110 m*M* NaCl, 5 m*M* KCl, and 1 m*M* $MgCl_2$, 2 °C) (= buffer A).The resulting pellet was resuspended in 3 mL with cold buffer A. After addition of 0.3 mL of 5 (and 6)-carboxyfluorescein diacetate (10 m*M* dimethylsulfoxide solution) (5 (6) CFD; C-8166, Sigma

Authors: Dipl.-Ing. Frithjof Thiele, Prof. Dr.-Ing. Werner Back, Lehrstuhl für Technologie der Brauerei I, Weihenstephaner Steig 20, 85354 Freising, Germany

Chemicals Co., St. Louis, MO/USA) the suspension was immediately shaken vigorously for 1 min and kept in an ice bath. After about 15 min, it was shaken again and allowed to stand for another 15 min. Yeast loaded with 5 (and 6)-carboxyfluorescein was washed three times with cold buffer A. The resulting pellet was resuspended in fresh cold buffer A and the suspension was filled up to 4 mL with the same buffer. Some 0.3 mL of this suspension was added to 8 mL of cold buffer A. After centrifugation, the resulting pellet was washed twice with 8 mL buffer A and resuspended in 8 mL cold buffer A. The suspension was kept for 90 min in an ice bath. During this period, the suspension was shaken about every 30 min to avoid cell sedimentation. After centrifugation, the resulting pellet was resuspended in 3.0 mL of buffer A at room temperature. The suspension was transferred to a quartz cuvette and the fluorescent intensity measured by spectrofluorophotometer (Kontron Instruments SFM 25, Bale/Switzerland) (emission wavelength 518 nm, excitation wavelengths 441 and 488 nm)(2).

2.3 Short ICP method (SICP)

The yeast slurry was filtered with a wire netting (400 mesh) to remove precipitates. A 2-mL yeast pellet was collected from pitching by centrifugation (2000 x g for 3 min at 2 °C). All centrifugations were carried out under these conditions, unless otherwise mentioned. The pellet was washed three times with cold 8 mL of a 50 m*M* citric disodium hydrogen phosphate buffer (pH 3.0, containing 110 m*M* NaCl, 5 m*M* KCl, and 1 m*M* $MgCl_2$, 2 °C) (= buffer A).The final pellet was resuspended in 3 mL of cold buffer A. After addition of 0.3 mL of 5 (and 6)-carboxyfluorescein diacetate (10 m*M* dimethylsulfoxide solution) (5 (6) CFD; C-8166, Sigma Chemicals Co., St. Louis, MO/USA) the suspension was immediately shaken vigorously for 1 min and 0,250 mL of this suspension was added to 8 mL of buffer A (duplicate) and this suspension was placed immediately in a water bath of 30 °C and incubated for 15 min.

After the 15 min incubation time the yeast was centrifuged and washed 2 times with cold buffer A. After the last centrifugation the yeast was resuspended in 3 mL of the same buffer and placed in a quartz cuvette and the fluorescent intensity measured by spectrofluorophotometer (Kontron Instruments SFM 25, Bale/Switzerland) (emission wavelength 518 nm, excitation wavelengths 441 and 488 nm).

2.4 Intracellular pH estimation of both methods

A calibration curve was determined by using 5 (and 6)-carboxyfluorescein in the indicated pH buffer (50 mM Citric disodium hydrogen phosphate buffer containing 110 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$; pH 6.6, 6.4, 6.2, 6.0, 5.8, 5.6, 5.4, 5.2, 5.0). It was constructed by plotting the ratio of fluorescent intensities (emission wavelength 518 nm) at excitation wavelengths of 488 and 441 nm as a function of pH. Intracellular pH was calculated by this calibration curve (2).

3 Results and discussion

3.1 Changes in the method

In the introduction it was already discussed that low temperatures used in the original method are important for reliable results but these temperatures causes the long sample preparation times. For a shorter method higher temperatures have to be applied, but without activating the cell metabolism in a way that the dye is translocated to the vacuoles. According to the literature other authors who worked with the same dye used incubation temperatures of 30 to 35 °C and 15 to 30 min incubation time (31,32). Breeuwer and Abee reported that in energized cells the Carboxyfluorecein was completely translocated to the vacuoles within approximately 20 min and exported to the extracellular environment in about one hour (31). This does not directly apply for the ICP method since only resting cells are measured not energized cells. The absence of sugars to energize the cells is ensured by several washing steps. But to avoid any translocation of dye under any circumstances an immediate measurement of the fluorescence after the incubation would be a possible application.

From this information the alternative sample preparation was derived: After 3 washing steps the yeast sample was incubated for 15 min at 30 °C with Carboxyfluorecein followed by 2 further washing steps in cold buffer and immediate measurement of the fluorescence. Variations in procedure, dye concentration and equipment settings have been tried but were not as successful (data not shown).

3.2 Reproducibility

The reproducibility is one of the most important parameters of a method. Imai et al found the excellent coefficient of variance of 0.40 % for the original method. To determine the reproducibility for the SICP method compared to the original method 8 individual analysis of each method from one batch of yeast has been executed. To get a complete overview this experiment has been done with yeast in very good physiological condition and with yeast in poor physiological condition. The results are presented in Table 1. The coefficient of variance has been found for both methods at about 0.8 % with the exception of the short method with bad yeast. Here a coefficient of variance of 1.7 % was found. This is still a very good value. This demonstrates that both methods are comparable and highly reproducible.

Table 1 Statistical parameters of a series of 8 individual experiments of each method executed with an active yeast and with a less active yeast

	Mean Value	Standard Deviation	Coefficient of Variation [%]	Confidence Interval 99 %
	active yeast			
ICP	6,43 pH	0,053	0,8	0,06
ICP Short-Method	6,75 pH	0,047	0,7	0,05
	less activ yeast			
ICP	5,46 pH	0,050	0,9	0,05
ICP Short-Method	5,48 pH	0,092	1,7	0,10

3.3 Correlation between the methods

For the comparison of the methods the physiological condition of different yeast batches have been analysed with both applications procedures and the R-squared values of the correlation have been determined. In Fig. 1 the correlation of all analyses of all four yeast strains are presented.

The results of the ICP method and the SICP method are not

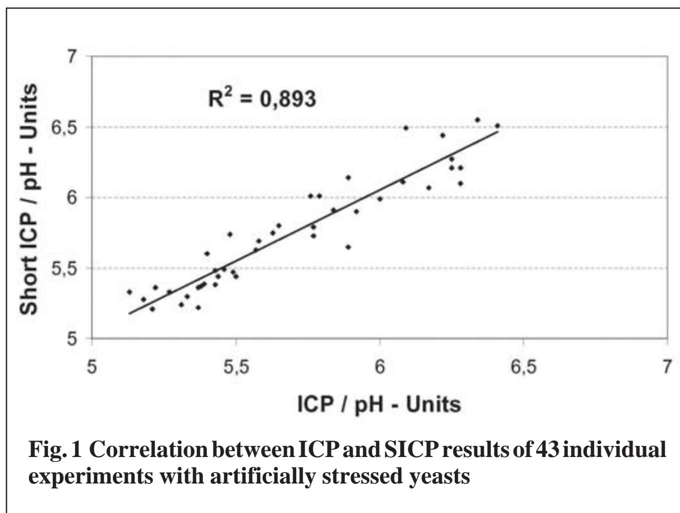

Fig. 1 Correlation between ICP and SICP results of 43 individual experiments with artificially stressed yeasts

identical but the R-squared value of 0.893 indicates a good correlation between the methods with a total number of 43 analyses. The R-squared values separated by the individual yeast strains are even higher. These values range between 0.89 and 0.99.

The different yeast conditions where obtained by applying different kind of stresses to yeast batches. This does not represent regular brewery conditions. But before this series of test have been run with different yeast strains the two methods have been used parallel for several month to evaluate whether the SICP method gives reasonable results or not. The tested yeasts included batches from different breweries, propagated yeast, cropped yeast and surplus yeast. The correlation of 55 analyses is presented in Fig. 2.

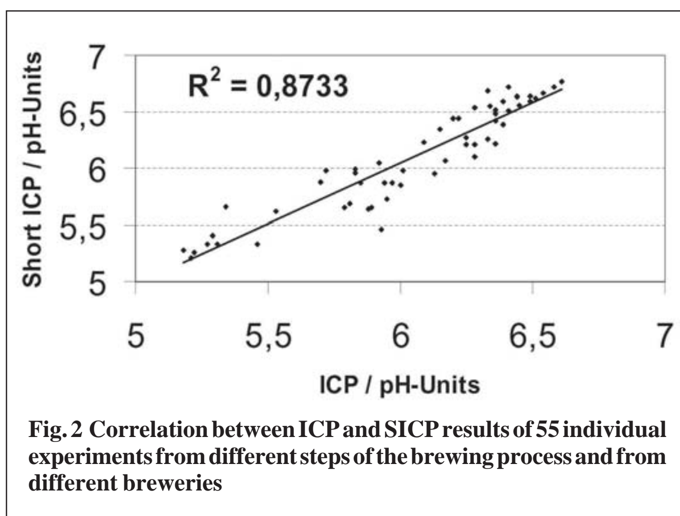

Fig. 2 Correlation between ICP and SICP results of 55 individual experiments from different steps of the brewing process and from different breweries

Since the results of both methods are not identical the question arise which is the correct intracellular pH or are both pHs correct. A possible explanation for the latter assumption is that the higher incubation temperatures result in a change of metabolism which leads to different pH values. The observation that very active cells show higher SICP values and less active cells tend to show lower SICP values than ICP values support this theory. Yeast cells in good physiological condition are activated by the higher incubation temperature while cells in poor condition are further weakened.

4 Conclusion

In various studies the ICP method has proofed to be a reliable and sensitive indicator for the yeast vitality. The labour intensive and long sample preparation has kept the method from being used in brewery laboratories. The Short ICP method cuts down the preparation time and the workload significantly. The results of the reproducibility tests and the correlations of the two methods indicate that the short ICP method is equally reproducible and comparable. This enables to determine the important parameter yeast vitality in a reliable way on a routine base.

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