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Scientific Evaluation of Different Methods for the Determination of Yeast Vitality

In this paper, two non standard methods are introduced and described precisely for measuring the vitality of yeast. Both are simple and produce results in a very short time. For the purpose of demonstrating these methods, brewing yeasts were placed under specific types of stress in various situations and compared to yeast not under stress. Their capacity for producing CO_2 was measured, and the condition of their cell walls was determined parallel through potentiometric titration. The results show that both of the methods demonstrated in this paper are effective for determining the physiological condition of yeast. Relative to the untreated yeast, the yeast placed under stress produced less CO_2 in controlled small-scale fermentation trials. Also, the condition of their cell walls changed, which was made apparent by the volume titrant required in the potentiometric titration. It should also be mentioned, that the trials described here were in fact preliminary trials for establishing the methodology for the respective tests. In a future paper, a further test designed for practical application in the industry includes a measurement of the intracellular pH and fermentation pressure in addition to the other measurements performed here.

Desricptors: yeast, brewing yeast, vitality, viability, physiological condition, CO_2, CO_2 production, pressure, temperature, potentiometric titration, cell wall surface area, surface charge

1 Introduction

Almost all foods prepared for human consumption undergo fermentative processes in order to transform or refine them in some way [13]. Measuring the vitality and viability of the microorganisms involved in these processes is therefore indispensible. While viability, at least of larger bacteria and yeast, is relatively simple to determine using staining methods [6], directly characterizing the physiological condition of these microbes requires a significantly amount of time and equipment. The following have become established as methods for determining the vitality of cells [20]:

- Measurement of the intracellular pH,
- acidification Power Test,
- vitaltitration,
- measurement of the capacity for metabolic processes (e.g. diacetyl reduction),
- determination of the quantity of certain metabolic products,
- vitality tests on the basis of benzoquinone and tetrazolium salts.

While results using these techniques are reproducible, at present these kinds of tests are not normally carried out in small and middle-sized facilities because of their complexity. Given the current situation, the challenge is to develop a novel method, one which is rapid, inexpensive, practical, suitable for everyday laboratory testing and delivers dependable results. A comparative analysis is included below, although the basis of this investigation is to determine the properties of the cell surface of bottom-fermenting yeast and to measure their capacity for CO_2 production.

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Figures see Appendix

2 General information

For the fermentation processes in a brewery, it is imperative that data regarding the vitality and the viability of the yeast used in the brewery be obtained on a regular basis, since the process and ultimately the product itself are, in large part, shaped by the physiological condition of the microorganisms.

2.1 Yeast viability

Yeast viability provides information about the ratio of living to dead yeast cells. The viability is important, because dead cells negatively affect the fermentation performance. Determining yeast viability is primarily accomplished using staining methods, for instance using methylene blue, or methods with fluorescent dyes, such as acridine orange, berberine and oxonol. The fluorescent dyes also seem to be very dependable but can be quite expensive as well as toxic. Brewing yeast should at least contain 95 % viable cells in order to be suitable for the brewing process [4].

2.2 Yeast vitality

Yeast vitality describes the physiological condition of the yeast, that is, its activity and fermentation performance. Analytically speaking, characterizing the vitality of the yeast is complicated and generally requires expensive equipment and/or sophisticated analysis procedures. Technical and process-related difficulties arise when yeast vitality deteriorates, which can lead to a decline

in product quality; for example, the sum of the medium fatty acid chains increases quantitatively as the yeast vitality drops. This, in turn, detrimentally affects the foam stability of the beer. Furthermore, good yeast vitality is essential for the color of the beer, especially for light-colored beers, as melanoid substances and polyphenol-protein compounds are normally taken up by the yeast over the course of fermentation, thus lightening the color. Additionally, poor yeast vitality leads to a significant increase in the concentration of the vicinal diketone diacetyl [5]. Two new, simplified and inexpensive methods have been developed for determining yeast vitality, and they are presented below.

2.3 Yeast strain TUM 34/70

For establishing a methodology, the yeast strain TUM 34/70 was chosen, because this yeast has been employed in previous scientific studies and has proven itself worthy as a reference strain for evaluating the physiological condition of yeast. Also, TUM 34/70 is probably the most widely used strain in the brewing industry at this time and is, not surprisingly, a very popular strain. *Wagner* described the strain as follows: "The yeast strain 34/70 is considered highly fermentative, exhibits optimal flocculation properties, which results in an excellent yeast harvest, reduces diacetyl very well, produces a moderate amount of higher alcohols, while the formation of esters is quite pronounced and the resulting beers are always of an outstanding quality" [24]. *Thiele* confirmed this view and specifically referred to the optimal SO_2 excretion, which as a rule results in a longer shelf-life and an enhanced flavor stability [20, p. 139].

2.4 Sample preparation for the experimental trials

Methods for determining the physiological condition of yeast provide a snapshot of the yeast's condition at the time of the test. Therefore, this kind of test should be carried out in a reproducible fashion at a number of different stages in the process; however, the concentration of the microorganisms can be very high, e.g. in the propagator, when harvested yeast undergo treatment and in the cone of the cylindro-conical tank (CCT), or it can be quite low, e.g. in the green beer or in the finished product. For this reason, a gentle method for increasing the number of yeast cells must be employed if the concentration is low. In order to adequately ensure that the yeast is treated in a gentle manner, the samples were centrifuged at cool temperatures at a low rotation velocity (5 min, 4000 rpm, 5 °C). Afterwards, the center of the sediment was removed (using a spatula) and gently stirred into a saline solution of a defined concentration. In our case, the salt mixture was prepared as a physiological saline solution known as "peptone water 0.1 %" (8.5 g NaCl, 1.0 g peptone derived from meat), which is used in microbiological preparations to maintain the osmotic pressure. The salt and peptone were dissolved in a defined volume of double-distilled and sterilized water accordingly to the description. An IKA Combimag Reo magnetic stirrer was utilized to homogenize the solution. After washing the yeast, a procedure intended to eliminate the negative effects of substances that could interfere with the analysis, the solution was centrifuged a second time for 5 min at 4000 rpm and 5 °C. A yeast sample was removed from the center of the sediment and the centrifugation procedure was repeated again. The remaining sediment was used in the analyses described here but not before the ratio of living to dead cells was determined by means of methylene blue staining and recorded for each sample [3].

The method for sample preparation described here proved effective and was therefore used to prepare the yeast samples for both the unstressed reference samples of yeast as well as those subjected to stress according to Thiele.

2.5 Purposefully influencing the physiological condition of the yeast

Because the methodology of the measurements was established during the trials described here, the physiological condition of the vital yeast was influenced in defined ways according to the methods developed by Thiele. These kinds of stress tests may not seem overtly relevant for brewing practices; however, they are valid tests, since they lead to a change in the intracellular pH, which is advantageous in scientific investigations, without altering the viability of the yeast to any significant degree. The viability of the stressed yeast often deviated only slightly from the yeast not subjected to stress.

Altering the physiological condition of the yeast according to Thiele was carried out as follows [20, p. 77-78]:

- **Ethanol stress:** In this case, the yeast was stressed through the addition of either a 1 % or a 10 % solution (% by weight) of 96 % ethanol derived from wine. The samples were placed in an incubator at a constant temperature of 36 °C for one hour. Stressing the yeast with a 1 % solution was carried out, contrary to the instructions given by Thiele, in addition to the stress test at 10 %, since during the preliminary trials it was determined that attempting to measure the yeast's ability to withstand stress at higher alcohol concentrations cannot be measured accurately.
- **Oxidative stress:** The yeast was stressed using 0.1 % and 1 % hydrogen peroxide (both % by weight). The samples were homogenized and allowed to stand for one hour at 24 °C.
- **Osmotic stress:** First, solutions of sorbitol at 15 % and a 20 % by weight were prepared, and then these solutions were mixed with the yeast and allowed to stand for 48 hours at 24 °C.
- **Nutrient deprivation**: The yeast samples intended for subsequent analysis were placed in double-distilled water and allowed to stand for one or three days at a constant temperature of 24 °C.

At the end of each stress test, the samples were subject to further treatment and prepared for the analyses as described above. The unstressed yeast serving as the reference samples were either cooled or were stored under identical conditions to the stressed samples for the duration of the test.

3 Preliminary tests and the measurement principle for evaluating yeast vitality by determining CO_2 production volumes

During alcoholic fermentation, ethanol, or acetaldehyde, is formed from pyruvate in glycolysis, which is then converted to ethanol and CO_2 by alcohol dehydrogenase.

150 years ago, *Balling* had quantitatively determined the amounts of ethanol, CO_2 and yeast produced as a result of the metabolic processes in a substrate undergoing fermentation. The following formula has reliably articulated this relationship since then:

$$2.0665\,\text{g Extract} \rightarrow \text{fermentation} \rightarrow 1\,\text{g ethanol} + 0.9565\,\text{g } CO_2 + 0.11\,\text{g yeast (dry matter)}$$

Because it has been shown that difficulties arise in performing small-scale fermentation tests with respect to their reproducibility, it was endeavored to develop the simplest possible format for these trials, one that could be easily and inexpensively recreated anywhere in the world. As a measure for the intensity and the rate of fermentation in these trials, the direct formation of CO_2 over time was chosen, since these methods of analysis require a considerably smaller sample volume than those for measuring the reduction in extract concentration. Moreover, rapid results can be expected by simply making provisions for adherence to the commensurate parameters. This is not the case with conventional methods. The measurements can also be carried out independent of others that may increase the likelihood of error.

In order to quantitatively determine the production of CO_2 in a reproducible manner, a compatible fermentation vessel was required that would be inexpensive and easily available around the world. Following exhaustive research and numerous preliminary tests, a choice was made: The Einhorn fermentation saccharometer. In 1905, *Dr. Einhorn* originally developed his unique glass tube to measure sugar in urine [3, p. 32], and today tubes of this design are available in various sizes from laboratory equipment supply companies. They normally cost between 15 and 20 EUR (20 to 30 USD) and offer the advantage of possessing defined graduations. Because of their diminutive size, they fit into most ordinary refrigerators and incubators without difficulty. Since Dr. Einhorn invented them, the automated nature of modern glassware production has allowed for a high level of reproducibility in their manufacture [11].

The properties of fluid dynamics pertaining to the fermentation vessel to be used for these trials also had to be factored into the choice. The Einhorn vessel can be divided into two areas, each exhibiting a different hydrostatic pressure: The taller portion is a closed, graduated tube down to the middle of the curve in the tube (on the left in figure 1), while the other portion is somewhat bulbous and opens upwards and outwards (on the right in figure 1). Some of the CO_2 produced during fermentation rises into both glass tubes and, if it passes through bulbous open tube, can simply disperse into the air, but the CO_2 trapped in the closed, graduated portion of the tube cannot, therefore causing the liquid at the bottom to be displaced. Consequently, the fill level rises in the open portion of the tube as the pressure builds in the closed portion.

In spite of modern glass manufacturing practices, the geometry of individual Einhorn tubes can vary slightly. The uncertainty regarding the effects of any slight variation in the hydrostatic pressure on the results due to minute differences in the geometry of the Einhorn tubes required that an investigation to be made as to whether it would be reasonable and necessary to release the CO_2 produced during fermentation at regular intervals.

Next, it was necessary that all of the tubes be compared to determine whether defined quantities of CO_2 are accurately displayed inside of each graduated tube in an identical manner. For this purpose, sterile wort was mixed with brewing yeast, and all of the tubes, which were numbered beforehand, were filled with homogenized green beer leaving no air trapped. Finally, the formation of CO_2 was measured at 10 min intervals at a constant room temperature, with the intention of creating a calibration curve for each tube. Figures 2 provides an example of one such curve.

The calibration curves were calculated for each tube under constant, identical conditions, taking into account each tube's individual gradations. All following test results refer to this calibration. Therefore, if for instance 100 % are reached, the CO_2 amount is identical to the CO_2 amount which was produced during the calibration in 120 minutes. The advantage of this calibration is that all curves which were generated showed a nearly linear behavior. Rogue results can therefore easily be identified. After the curves were finalized, the fermentation trials were performed as described above and the volume of CO_2 produced was recorded accordingly to the twelve-point-calibration. Over the course of the 80 min trials in a warmer environment (26 °C), some of the tubes were repeatedly turned upwards to allow the CO_2 to escape, thereby adding the CO_2 produced to the total up to that point, while the other once were left standing in the same position for the duration of the trial. A mean difference of 4 % between the two tubes in their total gas production was recorded for multiple trials (Fig. 3). Both exhibited similar fermentation profiles. The quicker fermentation in this experiment can be explained by the warmer fermentation temperatures (26 °C). Therefore the same amount of CO_2 which was produced during 120 min of calibration was produced here in 80 min. As figure 3 shows, this elaborate procedure appears to have been superfluous, as both fermentation trials were quite similar. The slight variation in the analysis results is due in all probability to distortion of the readings caused by foam as well as the additive effect of the reading errors as the trials progressed.

Because the goal of the procedures described here was to develop reproducible tests for yeast physiology on the basis of fermentation products, the substrate needed to be evaluated as well, in order to find the one best-suited for the specific nature of this application. Wort from brewery production tends to fluctuate in quality and is thus not suited for this purpose. Nutrients may be present in excess of what the yeast requires, they may leak. Fluctuations in the nutrients available in production wort make comparisons with other test series difficult; moreover, production wort is not always available and cannot be successfully stored for longer periods. For this reason, experiments were attempted with various sugars, available in pure form from laboratory supply companies. These sugars were dissolved in a defined amount of brewing water to produce an aqueous solution of 10 °Brix. Aside from the pure sugar, secondary wort products such as extracts and granulates were tested for their suitability. Figure 4 illustrates the various

fermentation trials, each of which was carried out using a comparable amount of yeast.

As can be seen in figure 4, fermentation proceeded at different rates depending on the kind of substrate employed. Numerous products appeared to be well-suited for this kind of analysis; however, a decision was made to continue the trials with sucrose, since it, unlike glucose, is inexpensive and easy to obtain worldwide and also possesses a long shelf-life. Sucrose is one of the sugars present in the wort, and one of the sugars that yeast take up rapidly at the beginning of primary fermentation [2, p. 71], while maltose, for example, must first be hydrolyzed intracellularly by the enzyme maltase to two glucose molecules [17]. For this reason, sucrose is advantageous as a substrate. Furthermore, weighing and diluting sucrose can be done gravimetrically in a simple and reproducible manner.

In a number of different preliminary trials, the amount of centrifuged yeast required was evaluated to ensure that a rapid and uniform fermentation in a 10 °Brix solution of sucrose ensues, so that ultimately the fermentation is able to be reproducibly characterized based on CO_2 production and subsequently represented visually in graph form.

The ideal amount of yeast emerged from weighing samples for the trials and consists of one part yeast centrifugate to two parts sugar solution, which resulted in an average cell count of 1.95×10^9 yeast cells/ml. Immediately after being weighed out, this mixture was placed on a magnetic stirrer (IKA Combimag Reo) for exactly 3 min, to homogenize and aerate the sample. Finally, a large amount of sugar solution ready for pitching was able to be prepared in a reproducible manner. After a short adaptation period (time required for regeneration) of 30 min, the yeast are again mixed well in the sugar solution and placed in the Einhorn tube free of gas and foam. The fill volume in each tube is chosen precisely, such that the closed portion is full of substrate to the curve, while the open, bulbous portion should remain as empty as possible. The temperature was held constant at 22 °C for all of the fermentation trials. Since the laboratories where the trials were performed are climate-controlled, the samples did not need to be placed in refrigerated storage or in an incubator.

As these preliminary tests have shown, measuring the production of gas can be realized relatively easily in a reproducible manner in analyses incorporating small-scale fermentation trials as long as certain parameters are taken into consideration.

4 Preliminary tests and the measurement principle for evaluating yeast vitality by determining their surface charge

In most cases, particles in solution possess a negative external charge [16]; this applies to brewing yeast as well [1]. The focus of this research was to determine if and to what extent the charge on the surface of a cell could be changed by purposefully influencing the physiological condition of the yeast. Furthermore, how precisely this change could be measured was also explored. The relatively new method of potentiometric titration was selected for conducting the tests. In scientific publications *Titze* has already shown that the charge on the surface of particles can be determined quantitatively using this method.

The measurement principle of potentiometric titration is based on the physical shift of the diffusely bound layer of ions on the surface [10, 19] and the changes in the potential measured during titration brought about by the introduction of particles possessing an opposing charge. Previous investigations by Titze have shown that this method can be employed to measure the current potential of particles as well as the surface charge density of particles in a solution [21, 22, 23].

The potentiometric titration apparatus consists of a measurement chamber and a plunger made of PTFE. A defined volume of yeast suspension must be transferred to the measurement chamber. Afterwards, the plunger is inserted into the filled chamber, so that a small space is formed along the wall. The yeast cells should be able to form a deposit on the wall of the chamber, thus creating a charged, diffuse layer [8, 9], which is presumably distributed relatively uniformly around each yeast cell. Next, the plunger is moved up and down in an oscillating manner. Due to the motion of the fluid, the cloud or concentration of charge is shifted resulting in a difference in electric potential which can be measured with the electrodes mounted on the upper rim and floor of the chamber. 10 µl of titrant is added to the yeast suspension via a dosing pump every three seconds until the electric potential has reached the zero point. The yeast suspension initially has a negative potential and is titrated with a positive polyelectrolyte solution of 0.001 n polyDADMAC, a substance capable of neutralizing the charge of the particles. As a result, fewer charged particles are attracted by the surrounding medium containing electrolytes. As the titration progresses, the overall electric potential of the cloud is reduced until it is dissipated when the neutral point is reached [22]. The titration is then stopped. In the instructions provided by the manufacturer, it is recommended that measurements be performed two to three times which has also been confirmed by the preliminary tests.

A potentiometric titration device manufactured by Particle Metrix GmbH, model Particlemetrix StabiSizer C, was used to determine the charge on the cell surface in the series of tests described in this paper. The same water (peptone water 0.1 %) used in sample preparation was selected as the medium for conducting the measurements. This water was used to wash the samples, in order to eliminate any substances that could potentially interfere with analysis. Due to its osmotic pressure, the water would not represent an additional source of stress for the yeast (refer to section 2.4 Sample preparation...).

In order to guarantee precise measurements, the medium was tested to establish whether and to what extent the measurements were reproducible. The data from multiple measurements of the medium are provided in figure 5.

The data plotted in figure 5 shows that for the five measurements performed, an average of 0.147 ml of titrant was required with a maximum deviation of 0.008 ml from the mean, although the initial value for the potential varied within the range of –108 and

–146 mV. The slight fluctuations in the values are most likely attributable to the fact that the volumetric flask is never completely dry because it is rinsed with the solution to be measured immediately prior to starting the measurement procedure. Therefore, there is always a slightly different fill volume in the flask, causing fluctuations in the measurement values to occur. In conclusion this medium is suitable for the dilution and preparation of additional samples, because it provides reproducible titration values and a precise titration of the zero point can be achieved.

In the next step, the optimal amount of yeast was determined experimentally. It was found that the final dilution of 1:100 (centrifuged yeast to water), which in our case was equivalent to a cell count of 3.85 x10^7 yeast cells/ml, yielded the most exact results (the sample preparation e.g. multiple centrifugation steps and washings etc., was identical to the preparation used for the CO_2 measurements). If the cell count in the yeast suspension is too high, there is a strong variation in the curves causing titration volumes to deviate greatly from the mean, thus eliminating any possibility of forming conclusions about the physiological condition of the yeast. Titze also advised against performing measurements in the presence of high particle concentrations because particles can agglomerate in media with high particle density thus creating larger particles. As a result, less titrant is required, because the total number of particles present is lower [21, p. 406]. However, if the yeast concentration is too low, as is the case with a dilution of 1:1000 as determined experimentally, the high salt content of the water and the resulting curve tend to become more dominant, approaching the values for a physiological sodium chloride solution.

Figure 6 shows a curve typical for a yeast suspension with 3.85 x 0^7 yeast cells/ml. The curve for a yeast suspension at the optimal dilution determined experimentally (1:100 dilution). The concentration of yeast cells was 3.85 x 10^7 per ml in the diluted suspension. Through evaluating the results it was found that the initial values measured for the potential of the different series of tests with the yeast samples are very close. The separation between the curves continues to shrink as titration progresses toward the zero point. However, there is a certain amount of scattering among the data for titration volume for the multiple measurements. The results from diverse preliminary tests showed, that the dispersion or scattering of data points around the mean increased as the total volume of titrant increased. The greater fluctuations associated with multiple measurements of the yeast suspensions as opposed to those performed with water containing higher saline concentrations can be attributed to the minimal differences in starting volumes, to slightly different yeast cell counts, and to certain deviations in the cell counts in the sample volume. Furthermore, the position of single cells in the measurement chamber during the analysis, the effects of agglomeration and the particles present in the medium also have an impact on the test results.

The preliminary test series demonstrated that yeast suspensions in the correct dilution can be measured in a reproducible fashion. A deviation of +/–0.03 ml of titrant from the calculated median of an unstressed suspension of yeast cells (3.85 x 10^7 yeast cells/ml) is acceptable as a realistic standard deviation and can be viewed as a tolerance range for the measurement value. However, should the volume of titrant increase to above 0.3 ml as it potentially could, in the case of a stressed yeast sample which contains broken cells and other particles, then larger deviations of up to +/–0.3 ml must be tolerated. In order to make a statement concerning the physiological condition of the yeast at a total titrant volume of up to 0.3 ml, the values from the tests should vary by at least 0.06 ml compared to the unstressed reference value. If more than 0.3 ml of titrant is required, then the intervals must be increased accordingly.

5 Results

After the basis of the reproducible measurements had been established for both methods as described above, the yeast samples were stressed and measurements were taken for both stressed and non-stressed organisms. In order to obtain a comparison of the two methods, the samples were prepared and analyzed in a group using both methods to ensure that cells of a similar physiological condition were being tested.

The following graphs illustrate the analysis results from this experiment. Vital bottom-fermenting yeast (TUM strain 34/70) collected from a fermenter, was used in the analysis; the same yeast, untreated, served as the reference. At this point, it must be mentioned that the slight differences in the values obtained from the reference measurements are most likely attributable to the fact that the yeast experienced a certain amount of aging over the analysis period. This in turn, led to differences in the physiological condition of the yeast samples, as it is evident in the figures of the viabilities. Of relevance here is never the less whether the recognizable differences between the measurements obtained from the treated and untreated yeast samples are measurable and if so to what degree they differ.

The results from both methods, one performed with an Einhorn fermentation saccharometer and the other using potentiometric titration are discussed below.

5.1 Ethanol stress

As described previously, yeast samples were subjected to stress through the addition of different amounts of ethanol. One portion of the yeast sample was mixed with 1 % ethanol prior to centrifugation, another portion of the yeast sample received 10 % ethanol and the third portion remained untreated, but was stored at the same temperature as the others. After a period of one hour, the samples were prepared by repeated rinsing and centrifugation as described.

Figure 7 clearly shows that the rate of fermentation is influenced according to the amount of ethanol in solution. Compared to unstressed yeast, which had a viability rate of 96 % in average, the sample with the addition of 1 % ethanol by weight produced almost 30 % less CO_2 during the same period, while the sample stressed by adding 10 % ethanol by weight produced 40 % less CO_2.

The determination of yeast vitality using the potentiometric method also exhibited significant differences, as illustrated by Figure 8. As illustrated, the fermentation curves of the stressed yeast samples exhibit a shift towards the right, meaning that it was

necessary to add a greater amount of titrant to achieve neutralization. Furthermore, the results depicted in the graph indicate, that a stronger level of yeast stress through ethanol results in a greater change in the charge on the cell surface (more negative charges). The yeast sample stressed with 1 % ethanol by weight required 0.237 ml of titrant, as opposed to 0.889 ml of titrant for the yeast stressed with 10 % ethanol by weight, corresponding to a factor of approximately 3.75.

5.2 Oxidative stress

In this series of tests, the yeast was stressed by adding 0.1 % H_2O_2 and 1 % H_2O_2 by weight, respectively. Figure 9 shows the results from the small-scale fermentation trials. As illustrated significant differences in the fermentation curves of the stressed and unstressed yeast samples can be measured, even so the viability of the used yeast was reduced to 94 %, due to aging, before the tests were performed. Almost 50 % less CO_2 was produced by the stressed yeast compared to the reference sample. However, a distinct difference between the concentrations of H_2O_2 was not detectable.

A similar pattern was observed in the measurements of the surface characteristics of the yeast cells by means of potentiometric titration (Fig. 10). The volume of titrant required for neutralization varied significantly after the yeast had been treated with H_2O_2. However, no correct, statistically significant difference between the two H_2O_2 concentrations was established.

5.3 Osmotic stress

The application of osmotic stress according to Thiele is carried out using Sorbitol. The yeast samples were stressed by subjecting them to 15 % and 20 % solutions of Sorbitol by weight. Due to aging the used yeast contained 92 % viable yeast cells. The results are pictured in figure 11. The fermentation profiles of both stressed yeast samples are practically identical. However, the values from the two stressed samples show close to a 70 % difference in CO_2 production compared to the unstressed reference sample.

Determining vitality via potentiometric titration (Fig. 12) yielded a similar result. As with the previous tests, the unstressed reference sample can readily be differentiated from the stressed samples. Only slight differences between the 15 % and 20 % solutions of sorbitol were observed, as with the results for the CO_2 production.

5.4 Nutrient deprivation

In this test series, nutrient deprivation was simulated by adding distilled water to the yeast samples. This type of stress proved to have less impact than other types as it is obvious from the results in figure 13. No significant measurable difference was apparent between the CO_2 produced by the treated and untreated yeast samples one day after the water was added. Even after a three day period of storage, only a difference of less than 14 % in median was recorded. The reason for this result may be, because the used yeast contained only 89 % viable yeast cells do to aging before it was treated accordingly. This effect will be discussed in the conclusion precisely.

The analysis of the physiological condition of the yeast by means of potentiometric titration showed a comparable trend. Figure 14 illustrates, that no significant differences were exhibited by the stressed and unstressed yeast samples of the same ages, independent of the duration of the stress.

6 Summary and interpretation

Results from the preliminary tests indicate that, with the methods and procedures described here, it is possible to determine the physiological condition of yeast. The different types of stress tests also showed that both methods can be applied to measure differences in physiological condition of yeast.

6.1 Ethanol stress

The tests yielded results which demonstrate that yeast subjected to ethanol stress not only exhibits a slower rate of fermentation, but changes in the properties of the surface of their cells as well. Furthermore, higher concentrations of alcohol have a stronger influence on the physiological condition of the yeast. These effects could be measured by both methods.

Interpretation: Ethanol is excreted by the yeast and has a toxic effect on the cells at higher concentrations [15]. Toxicity is even more pronounced if the concentration is suddenly increased artificially [7]. Generally speaking, the fact that ethanol stress on yeast can be measured so clearly can be explained by the change in the cell membrane due to exposure to ethanol. *Jimenez* and *Benitez* [12] demonstrated that the relationship of proteins and lipids in the membrane structure of yeast was modified through the presence of ethanol. Additionally, the increased concentration of the disaccharide trehalose formed by yeast in stress situations also affects the composition of the cell membrane, because it can be deposited on both the inside and outside of the cell membrane in order to stabilize it. [21, pp. 93, 111-112]. Therefore a change of the particle charge as well as slower fermentation speed, due to longer adaption times may result.

6.2 Oxidative stress

Significant differences were observed in the fermentation profiles of stressed and unstressed yeast samples as a result of the stress induced in the tests described previously. The concentration of H_2O_2, however, appeared to be less important. The fermentation performance was reduced by approximately 45 % in each case, as was visible from the amount of CO_2 produced. A difference between stressed and unstressed yeast cells was also evident in the potentiometric titration results. Again however, there was no significant difference between the concentrations of H_2O_2 applied.

Interpretation: In daily operations, yeast can be exposed to oxidative stress through excessive wort aeration. Radicals are formed through the induction of enzymes, which can then lead to the oxidation and destruction of cell components [1, p. 52]. Certainly the usage of H_2O_2 has a similar, but in comparison much stronger effect on the cells. The damage which is caused by 0,1 %

H_2O_2 seems to be nearly as significant as the effect which results using 1 % H_2O_2.

6.3 Osmotic stress

Distinct differences were apparent in the measurements taken in conjunction with the sorbitol test and the reference samples for both analysis methods. The rate of fermentation dropped to almost 65 % after the treatment with sorbitol, regardless of whether the concentration of the sorbitol solution was 15 % or 20 % by weight. A similar pattern was observed in the analyses conducted using potentiometric titration.

Interpretation: It has already been established that conditions during fermentation are not optimal when yeast are used to produce beers with a high original gravity. This is due to the increase in osmotic stress which can negatively affect yeast physiology [15, p.288].

It is apparent that the osmotic stress causes a distinct reaction in the interior of the cell compartment that alters the charge on the surface of the cell. Through treatment with sorbitol, the cell experiences hyperosmotic shock. As a consequence, water inside the cell diffuses to the exterior, causing the cell volume to shrink by 45 to 53 % [14]. For this reason, yeast cells accumulate glycerol and trehalose, primarily to equalize changes in osmotic pressure [18]. As mentioned in the section discussing the results of ethanol stress, an increase in trehalose content results in the stabilization of the cell membrane, which could also lead to an increase in the charge on the surface of the cell.

6.4 Nutrient deprivation

Research from Thiele has already established that nutrient deprivation occurring for a limited time appears to have only a slight effect on yeast physiology. Our here presented experiments verify these results. Therefore no clear effect due to nutrient deprivation could be measured with either of the two here presented methods.

Interpretation: Because yeast harvested from fermenters was used in this series of tests, and the yeast experienced a certain amount of aging during the tests, it can be assumed that the yeast was not in a medium considered to be particularly rich in nutrients. Naturally, this makes measurements based on the differences described here much more difficult, so that no clear differentiation devoted.

8 Discussion and future research

The results of the preliminary tests obtained using the two methods described in this paper have shown that the physiological condition of brewing yeast is a measureable parameter.

The focus of further tests will be to investigate (presented in a subsequent work) whether a simplified sample preparation procedure can be developed which will allow the physiological condition of the yeast to be determined with less effort.

Furthermore, additional test series will employ harvested yeast that has been subjected to the types of stress typically encountered in daily operations and the change in the physiological condition over time will be determined. In addition to testing using the methods described here, the inclusion of the ICP as well as a simple differential pressure test will be utilized.

In the future, the surface charge of various microorganisms will be characterized and the differences identified. Correlations between the physiological condition of brewing yeast and the formation of aroma components as well as aging indicators will be evaluated as well.

Acknowledgements

The authors would particularly like to thank the Bachelor students *Nicole Dünzer* and *Roman Werner*. Only through their reliable, precise and autonomous work could this project have been successfully completed.

9 Literature

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Received 05 September 2011, accepted 20 October, 2011

Appendix

Fig. 1 Einhorn fermentation saccharometer

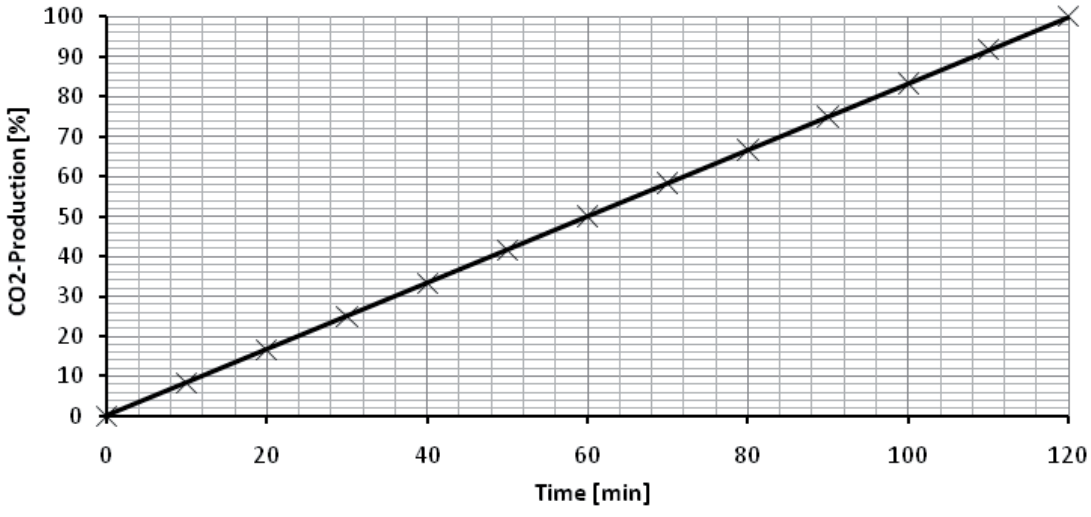

Fig. 2 Examplary calibration curve

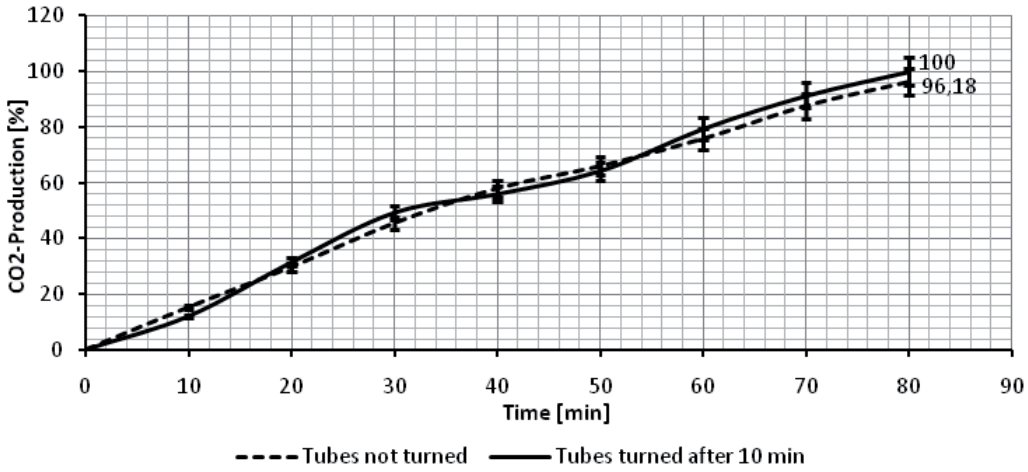

Fig. 3 Influence of different hydrostatic pressures in the Einhorn fermentation tubes

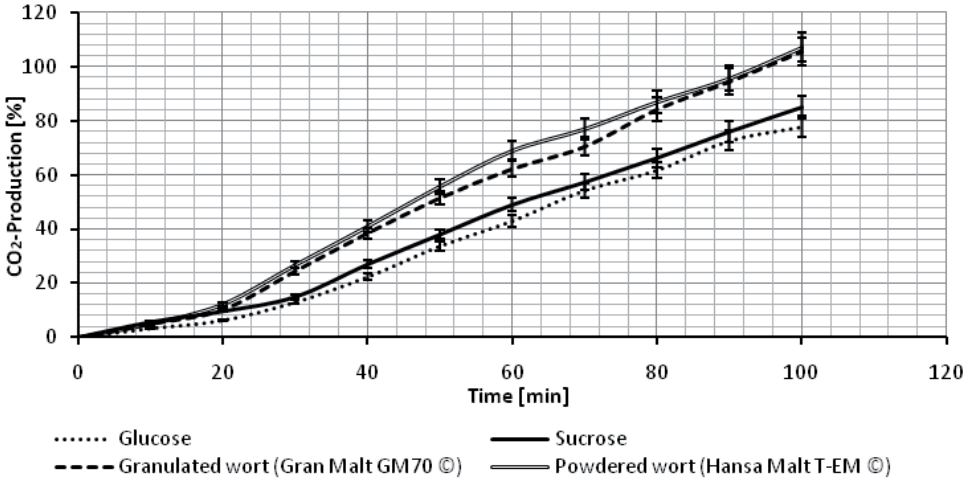

Fig. 4 Fermentation profiles for glucose, sucrose and secondary wort products

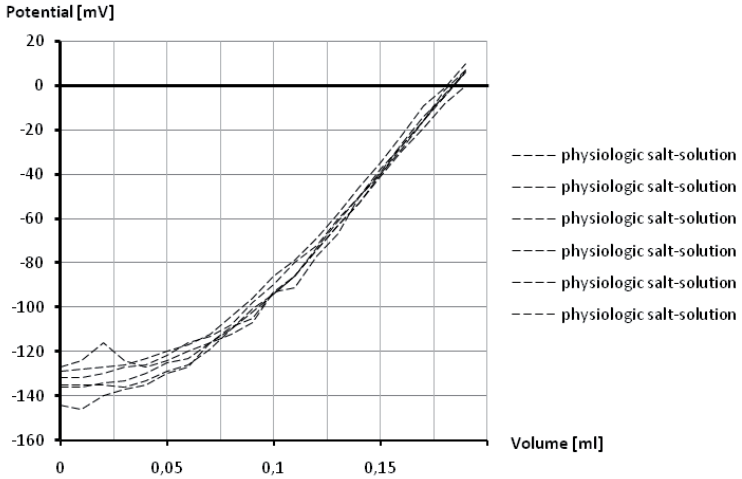

Fig. 5 Potentiometric titration profiles of salt solution

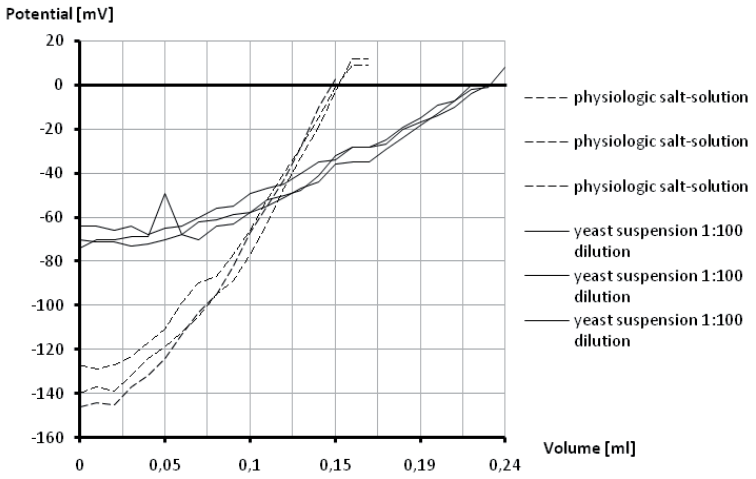

Fig. 6 Potentiometric titration profiles of yeast suspension, 1:100 dilution (≙ 3,85 x 10^7 yeast cells/ml)

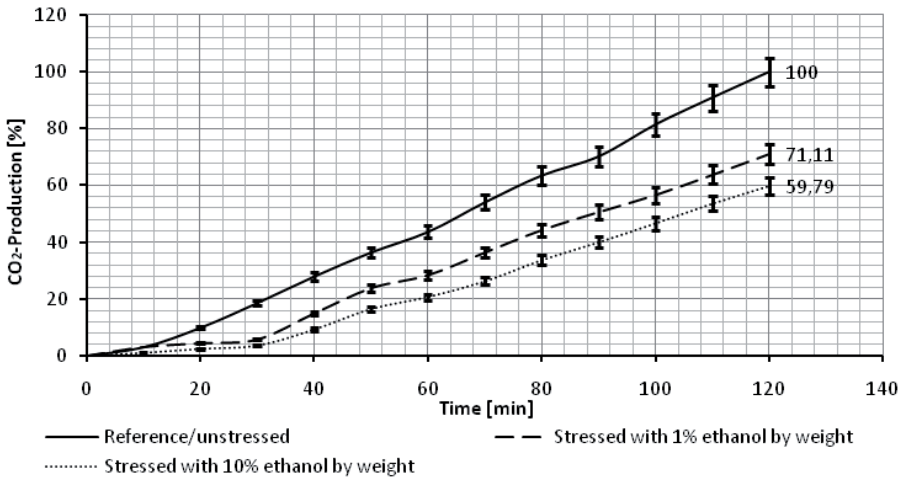

Fig. 7 Fermentation profiles of yeast subjected to ethanol stress

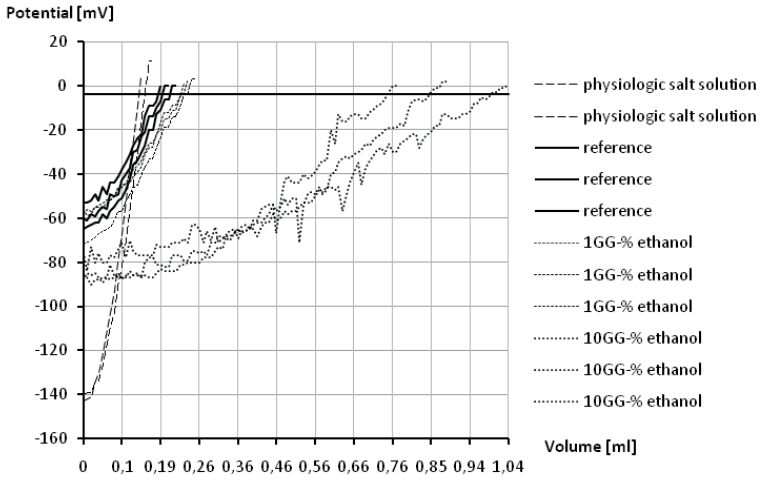

Fig. 8 Potentiometric titration profiles of yeast subjected to ethanol stress

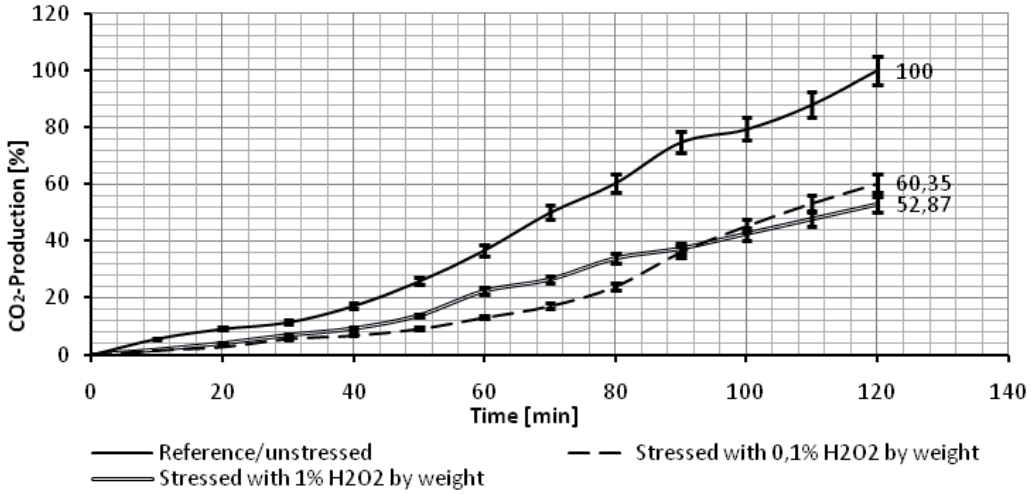

Fig. 9 Fermentation profiles of yeast subjected to oxidative stress

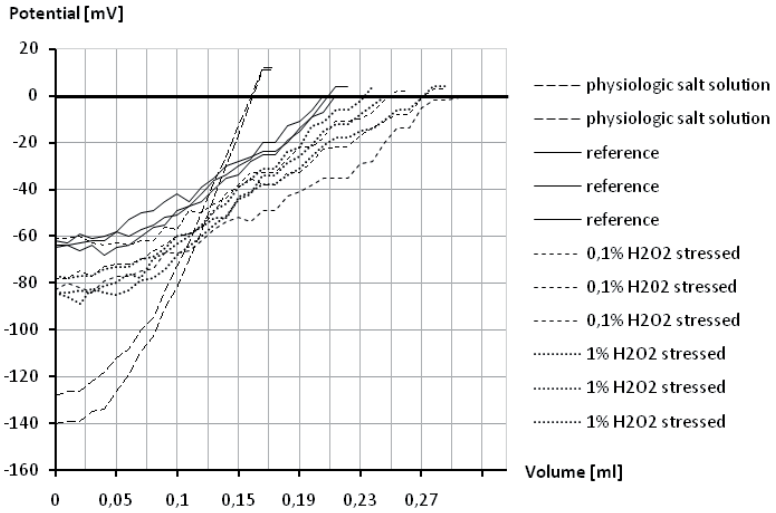

Fig. 10 Potentiometric titration profiles of yeast subjected to oxidative stress

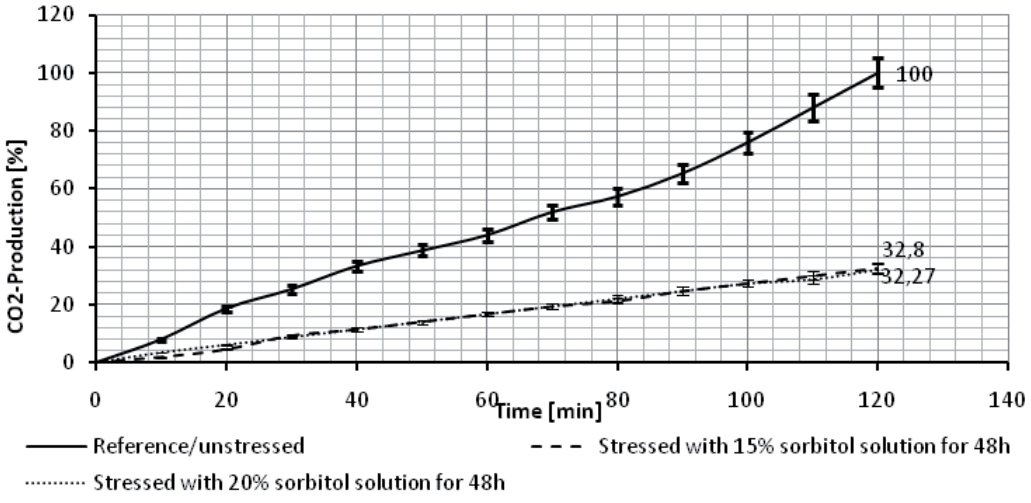

Fig. 11 Fermentation profiles of yeast subjected to osmotic stress

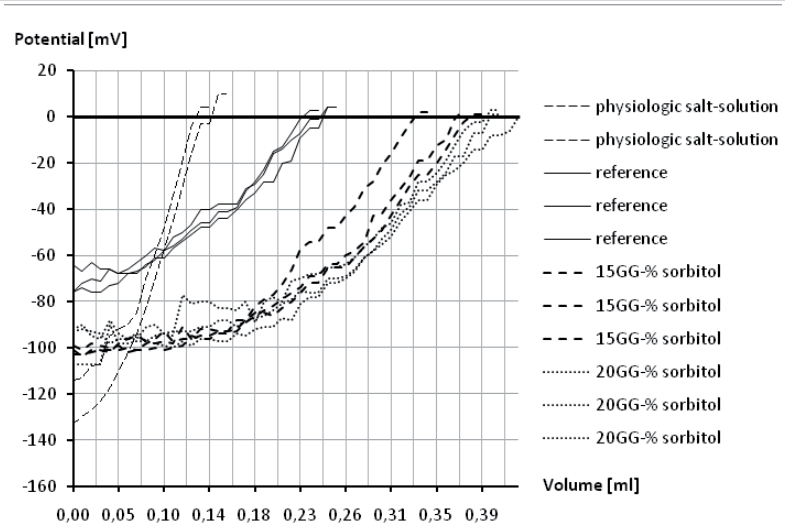

Fig. 12 Potentiometric titration profiles of yeast subjected to osmotic stress

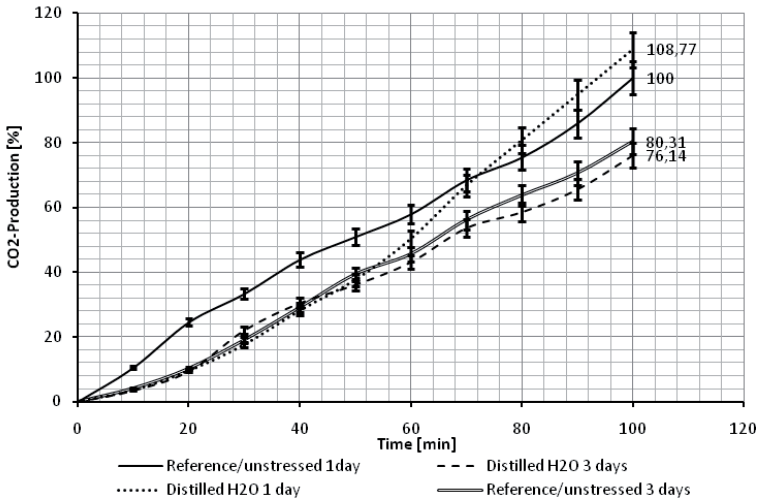

Fig. 13 Fermentation profiles of yeast subjected to nutrient deprivation

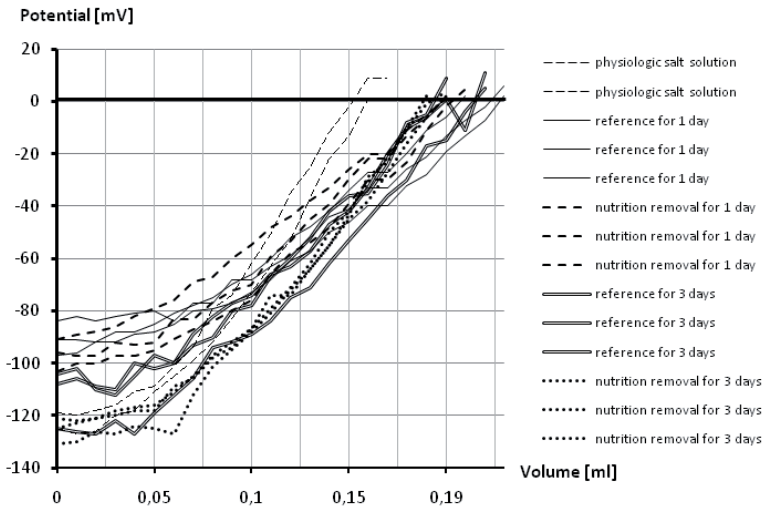

Fig. 14 Potentiometric titration of yeast subjected to nutrient deprivation