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New Concept for Physiological Assessment of Yeast Cells

At present, there are various methods for evaluating yeast viability and vitality. Yeast viability is a measure of the ratio of dead cells and yeast vitality is a measure of the vigor of living cells. All of the methods that evaluate yeast physiological condition measure only viability or vitality. Under real-world conditions such as actual brewing, however, viability and vitality are often unrelated. As a result, there is a fundamental problem with the current evaluation methods for vitality and viability. It is necessary to simultaneously observe both the dead cell ratio and vitality of living cells in order to precisely evaluate the physiological state of yeast. However, there have been no methods developed to resolve this fundamental problem to date. In this paper, a new method for simultaneously evaluating both viability and vitality is proposed using recent advances in flow cytometry; TO-PRO3 staining was applied for evaluation of viability and intracellular pH was applied for evaluation of vitality. First, the intracellular pH of yeast cells was measured using an argon laser, and the cell was determined to be dead or alive using a He/Ne laser. This new method was reproducible and could evaluate the subtle differences in viability and vitality that affect fermentation performance and beer quality. As this method could easily and accurately evaluate both viability and vitality of individual cells simultaneously, the fundamental problems with current evaluation methods were resolved. The simultaneous evaluation of both viability and vitality is essential for the brewing of quality beer.

Descriptors: yeast, vitality, viability, simultaneous evaluation, flow cytometer, intracellular pH, TO-PRO3

1 Introduction

The quality (viability and vitality) of pitching yeast is a very important factor in maintaining a consistent fermentation performance to give a beer of acceptable quality [1, 3, 12, 16, 19, 24, 27, 33, 34, 37]. Therefore, to brew quality beers, various methods for the determination of yeast viability and vitality have been developed, including staining methods, cell replication methods and methods based on physiological parameters [1, 4, 5, 6, 7, 9, 11, 13, 15, 17, 18, 20, 22, 25, 26, 27, 35]. However, the concepts of vitality and viability are quite different. Yeast viability essentially divides dead cells from other cells, while yeast vitality is simply a measure of the vigor of living cells (Figure 1) [12, 21]. Previously, yeast vitality was assessed using the cell mass, for example, by using fermentation tests, acidification power tests, and measures of oxygen uptake or CO_2 production. At present, cell biology has progressed and individual yeast cell vitality can now be analyzed precisely [14, 15, 16, 32, 34, 35, 36]. Nonetheless, new problems have developed because yeast vitality measures only living cells and yeast viability measures only dead cells.

Originally, we have to simultaneously observe both the dead cell ratio and the vitality of living cells, as shown in figure 1. When only viability (the dead cell ratio) is measured, it is difficult to estimate vitality using the data of viability as vitality is a measure of the vigor of living cells. Moreover, when only vitality is measured, it is difficult to estimate viability (dead cell ratio) using the data of vitality as viability (dead cell ratio) is a measure of dead cells. There are many cases in which both the dead cell ratio and vitality are low,

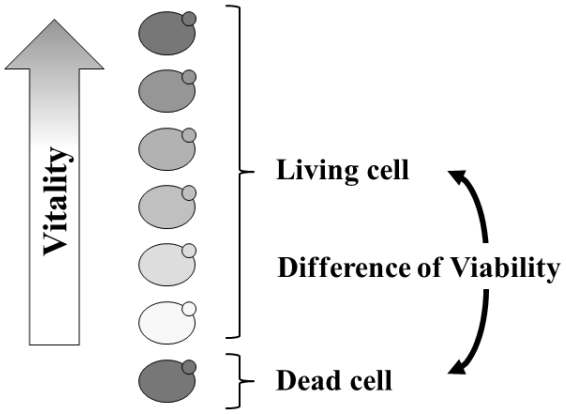

Fig. 1 Difference between viability and vitality [12, 21].

Yeast viability is a measure of the ratio of dead cells and yeast vitality is a measure of the vigor of living cells. To assess the physiological condition of a yeast population precisely, it is essential to evaluate all the cells shown in this figure, that is both dead and living cells

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or high. These situations can arise depending on yeast handling, fermentation, wort preparation and raw materials. A fundamental problem with the current methods for evaluating vitality and viability is that it is difficult to simultaneously evaluate both viability and vitality, but recent advances in flow cytometry have enabled us to develop a new method to resolve this issue.

In this paper, we propose a new concept for the simultaneous, sensitive and simple evaluation of both viability and vitality in individual yeast cells using a flow cytometer.

2 Materials and methods

2.1 Simultaneous measurement of yeast viability and vitality

In this new method, the fluorescence method, which is based on the membrane permeability of dead cells, was applied to the evaluation of viability and the intracellular pH (ICP) method [17], which can evaluate subtle difference of yeast vitality, was applied to the evaluation of vitality. In addition, 100 mM 5(and 6)-carboxy fluorescein diacetate was used instead of 10 mM 5(and 6)-carboxy fluorescein diacetate, which was described in [17]. As the fluorescent dye for viability measurement, TO-PRO3 was selected because TO-PRO3 and carboxy fluorescein do not interfere with one another during specific fluorescence measurement by flow cytometry.

The bottom-fermenting yeast slurry from brewery conditions was filtered with wire netting (400 mesh) to remove precipitates. Next, 2 ml of yeast pellet collected by centrifugation (3,000 × g, 5 min, 2 °C: centrifugation was carried out under these conditions, unless otherwise noted) was washed twice with 50 mM MES buffer (pH 6.20, containing 110 mM NaCl, 5 mM KCl and 1 mM $MgCl_2$, 1 °C).

The final pellet was resuspended in cold MES buffer and the suspension was made up to 3 ml with the same buffer. A total of 30 µL of 5 (and 6)-carboxy fluorescein diacetate (100 mM dimethylsulfoxide solution; Wako Pure Chemical Industries) and 30 µL of TO-PRO3 (1 mM dimethylsulfoxide solution; Invitrogen) were added to the yeast suspension, followed by vigorous shaking for 1 min to mix the contents. The suspension was kept in an ice bath. After about 15 min, it was mixed again and kept for another 15 min. The stained yeast was washed three times with cold 50 mM citrate/disodium hydrogen phosphate buffer (pH 3.0, containing 110 mM NaCl, 5 mM KCl and 1 mM $MgCl_2$). The resulting pellet was resuspended in fresh cold 50 mM citrate/disodium hydrogen phosphate buffer and the suspension was filled up to 4 ml with the same buffer.

Of this suspension, 0.3 ml was added to 10 ml of cold 50 µM citrate/disodium hydrogen phosphate buffer. After centrifugation, the resulting pellet was washed with cold citrate/disodium hydrogen phosphate buffer twice, and was resuspended with 10 ml of the same cold buffer. This suspension was kept for 90 min in an ice bath (during this period, the suspension was mixed every 30 min to avoid cell sedimentation). After centrifugation, the resulting pellet was resuspended with 3.0 ml of cold 50 mM citrate/disodium hydrogen phosphate buffer. A total of 50 mL of this suspension

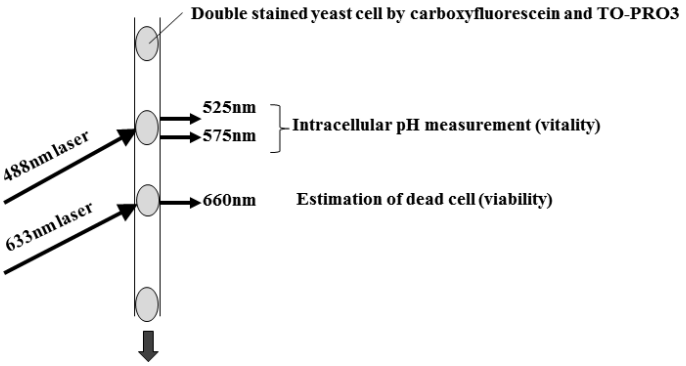

Fig. 2 Principle of a new method for simultaneous measurement of visability and vitality using flowcytometer.

Double stained yeast cell by both carboxyfluorescein (for intracellular pH measurement) and TO-PRO3 (for dead cell detection is first analyzed by 488 nm laser for vitality and then analyzed by 630 nm laser for viability

was added to a sample tube (for flow cytometer; Becton Dikinson LSRII) containing 1 mL of cold 50 mM citrate/disodium hydrogen phosphate buffer. The flow rate was 200 cells/second and 30,000 cells/sample were analyzed by flow cytometry. Intracellular pH was measured using a 488-nm argon laser based on fluorescence intensities at 525 nm and 575 nm. Yeast cells stained with TO-PRO3 were detected using a 633 nm He/Ne laser (emission wavelength = 660 nm) (Figure 2).

2.2 Calibration for determination of pH

Non-fluorescent particles (SuperdexTM 200; Amersham Pharmacia Biotech AG) were equivalent to the indicated pH buffer (50 mM citrate/disodium hydrogen phosphate buffer; pH 3.0–7.0) containing 5(6)-carboxyfluorescein (Wako Pure Chemical Industries). Fluorescence intensities at 525 nm and 575 nm were measured by the 488-nm argon laser in the flow cytometer.

2.3 Fermentation

Fermentation was carried out at 12 °C in 0.5-L tall tubes (125 cm × 2.5 cm i.d.) for 7 days. Air-saturated wort (13.5 °P) was inoculated with 20 million cells/ml yeast. Specific gravity was monitored at intervals during fermentation using the Anton Paar DMA 4500.

2.4 Preparation of yeast samples containing different content of dead yeast cells for the model experiment

Dead yeast cells without intracellular esterase activity were prepared as follows. The bottom-fermenting yeast slurry from brewery conditions was filtered with wire netting (400 mesh) to remove precipitates. Yeast was washed twice with 50 mM Tris buffer containing 5 % sodium dodecyl sulphite (pH 9.0), and was resuspended in the same buffer and incubated for 15 minutes at 37 °C. Incubated yeast was washed 3 times with 50 mM Tris buffer containing 5 mM EDTA (pH 9.0), and was resuspended in the same buffer. To remove sodium dodecyl sulphite, yeast suspensions were rotated for two weeks at 20 °C, changing the buffer once a day.

This dead yeast was mixed with fresh yeast at different ratios (0 %–90 %).

Methylene blue staining for estimation of viability was then performed as described elsewhere [7].

2.5 Stored condition of yeast cells

The bottom-fermenting yeast slurry from brewery conditions was stored for 15 days at –2 °C, 2 °C or 6 °C in a cool bath using propylene glycol.

2.6 Reconstitution of plasma membrane ATPase into liposome for principle of ICP method

Plasma membrane ATPase from bottom-fermenting yeast was purified as described by Perlin [23]. As plasma membrane ATPase is a membrane protein that requires specific phospholipids [13], solubilized buffer was prepared using 10 mM HEPES-KOH (pH 7.2), 1 M KCl, 45 % glycerol, 0.2 mM EDTA, 1 mM DTT(dithiothreitol), 1 mg/ml phosphatidyl serine (Brain extract: Folch Fraction III; Sigma) and 1 mg/ml soy bean phospholipid (phosphatidyl choline typeII-S, Sigma). Next, 1 ml of purified plasma membrane ATPase solution in solubilized buffer containing 3 mg/ml phosphatidyl serine, 1.5 mg/ml soy bean phospholipid, 0.8 % octylglucoside was mixed with 1 ml of solubilized buffer, 90 µl of 50 mg/ml soy bean phospholipid, 540 µl of 50 mg/ml phosphatidyl serine. Octylglucoside was added to give a final concentration of 0.8 % (w/v), and this was left to stand for 5 minutes on ice. This solution was dropped gently into 25 ml of reconstitution buffer (10 mM HEPES-KOH (pH 6.8), 300 mM KCL, 1 mM DTT). After centrifugation (300,000 × g, 1 hour), reconstituted liposomes were resuspended in 300 µl of reconstitution buffer. A total of 7.5 µl of this reconstituted liposome was suspended in 600 ml of quenching buffer (10 mM HEPES (pH 6.8), 50 mM KCL, 1.67 µM ACMA (9-amino-6-chloro-2-methoxyacridine), followed by incubation at 25 °C. The reaction was started by addition of 5 mM ATP solution containing 5 mM $MgCl_2$. Orthovanadate (10 mM) was used as a potential inhibitor of plasma membrane ATPase, and 8 mM FCCP (carbonyl cyanide p-trifluoromethoxy phenylhydrazon) was used as the protonophore. Fluorescence intensity (excitation, 400 nm; emission, 460 nm) was measured using the RF-5000 (Shimazu).

3 Results and Discussion

3.1 H^+ transportation by purified plasma membrane ATPase of brewing yeast

Plasma membrane ATPase is essential for yeast growth [32]. This protein extrudes intracellular H^+ to the extracellular environment, forming a transmembrane H^+ gradient [31]. The transmembrane H^+ gradient is the driving force for uptake of Maltose [29] and amino acids [28], as shown in figure 3. In addition, intracellular pH regulates the key enzymes in glycolysis and gluconeogenesis. As the key enzymes in glycolysis and gluconeogenesis are regulated by a cascade reaction involving cyclic AMP (cAMP)-dependent protein kinases, cAMP plays an important role in this regulation. It was found that cAMP is regulated by intracellular pH. Moreover, intracellular pH is thought to be a trigger for other cell responses, including the induction of heat shock proteins. Thus, intracellular pH is considered to be an important factor in yeast physiology [15].

Using intracellular pH, a highly sensitive method (ICP method) for evaluation of yeast vitality was developed [17]. Figure 4 shows the relationship between intracellular pH and plasma membrane ATPase activity in brewing yeast [13]. Figure 5 (see next page) shows H^+ transportation by the plasma membrane ATPase of brewing yeast. Purified plasma membrane ATPase was reconstituted into liposomes of phosphatidylcholine and phosphatidyl serine, and H^+ transportation by the plasma membrane ATPase of brewing yeast was investigated. If plasma membrane ATPase shows H^+ transportation activity, plasma membrane ATPase reconstituted in the liposome would hydrolyze ATP outside of the liposome and H^+ would be transported into the liposome, thereby lowering the pH inside the liposome. This pH lowering activity can be detected by quenching of a fluorescent reagent (ACMA). As shown in figure 5, lowering of fluorescence intensity was observed by addition of ATP solution containing $MgCl_2$, thus confirming that the plasma membrane ATPase of brewing yeast transports H^+. Moreover, the decreased fluorescence intensity was elevated by addition of protonophore (FCCP). This suggests that the H^+ gradient formed

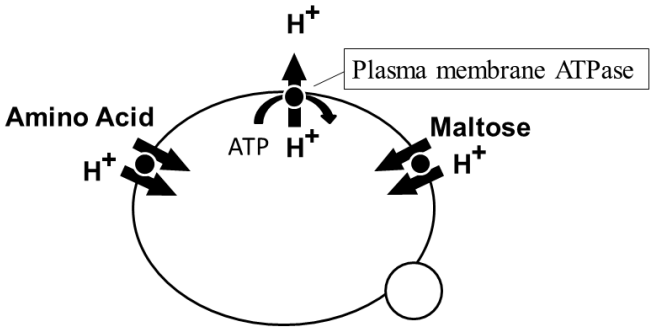

Fig. 3 **Transmembrane H^+ gradient which plasma membrane ATPase forms is the driving force for uptake of maltose and amino acids**

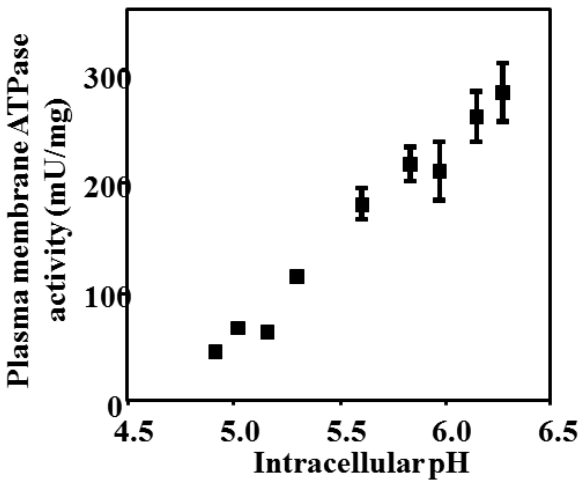

Fig. 4 **The relationship between intracellular pH and plasma membrane ATPase activity of brewing yeast [13].**

Various yeast samples were prepared by storing at 1°C. The intracellular pH by ICP method which measures intracellular pH of cell mass using fluorescent spectrophometer and plasma membrane ATPase activity of each samples were measured. Plasma membrane ATPase activity was assayed by the method described in [30]. The activity of 1U means to break down 1mmol ATP per one minute

between the outside and inside of the liposome was diminished by the protonophore. As shown in figure 5 (chart B), these phenomena were not observed when a specific inhibitor (orthovanadate) of plasma membrane ATPase was added before ATP solution containing $MgCl_2$. Taken together, these data confirm that the plasma membrane ATPase of brewing yeast transports H^+ and forms a H^+ gradient across the membrane.

The ICP method measures plasma membrane ATPase activity, which regulates intracellular pH. In the new method proposed in this paper, the ICP method was applied for evaluation of yeast vitality.

3.2 Calibration curve for determination of intracellular pH, dead cell ratio and reproducibility of this new method

Figure 6 shows the plots of pH versus the ratio of fluorescence intensities at 525 nm and 575 nm using a 488-nm argon laser. Using this calibration curve, the intracellular pH was calculated from the fluorescence intensities at 525 nm and 575 nm.

Figure 7 shows the estimation of dead cells using flow cytometry. Yeast cells with fluorescence intensities at 660 nm of more than 1×10^3 were considered to be dead, as the cells are stained with TO-PRO3.

Table 1 (see next page) shows the reproducibility of this method.

In the case of intracellular pH, the variation coefficients of the mean, median and standard deviation were 0.12%, 0.09% and 0.68%, respectively.

In the case of the dead cell ratio, the variation coefficient of the mean was 4.1%. In comparison with the methylene blue method, this new method using TO-PRO3 was reproducible. As shown in Table 1, the variation coefficient of this new method was much

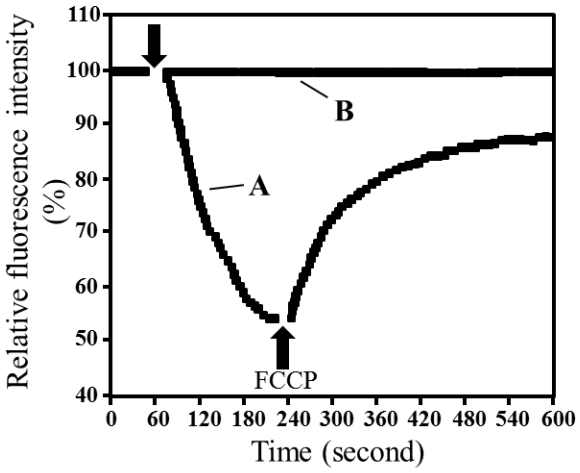

Fig. 5 H^+ transportation by plasma membrane ATPase of brewing yeast.

The purified plasma membrane ATPase reconstituted into liposome was used to observe ht H^+ transportation activity. The reaction was started at the 60 seconds by addition of ATP solution containing $MgCl_2$. In the case of chart A, the protonophore (FCCP) was added at the 240 seconds. In the case of chart B, the potential inhibitor (orthovanadate) of plasma membrane ATPase was added at 0 seconds

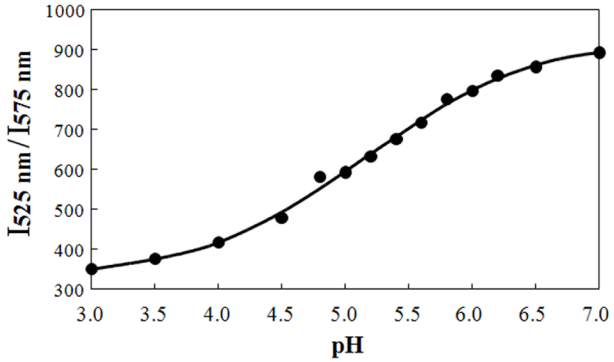

Fig. 6 Calibration curve for determination of intracellular pH.

I525 nm refers to fluorescence intensity at 525 nm by argon laser and I575 nm refers to fluorescence intesity at 575 nm by argon laser. Using this calibration curve, the intracellular pH of individual cells was calculated by dividing the value of I525 nm by that of I575

smaller than that of the methylene blue method (24%). Although the methylene blue method using microscopy is simple, this new method is also easy to perform, as 30,000 individual cells could be analyzed for both viability and vitality in a few minutes using the flow cytometer.

3.3 Viability and vitality evaluation of yeast samples with no correlation between viability and vitality (model experiment)

It is thought that the viability and vitality of cropped yeast show similar fluctuations. That is, when viability is low, vitality is also low, and vice versa. Therefore, measurement of either viability or vitality alone has been considered to be sufficient for evaluating yeast physiological status. However, there is not always a clear relationship between the viability and vitality of yeast samples. We first investigated whether the new method proposed in this paper could assess yeast samples containing different proportions of dead cells in the same vital cell populations.

In order to assess whether this new method works, dead cells without intracellular esterase activity were prepared, as described in

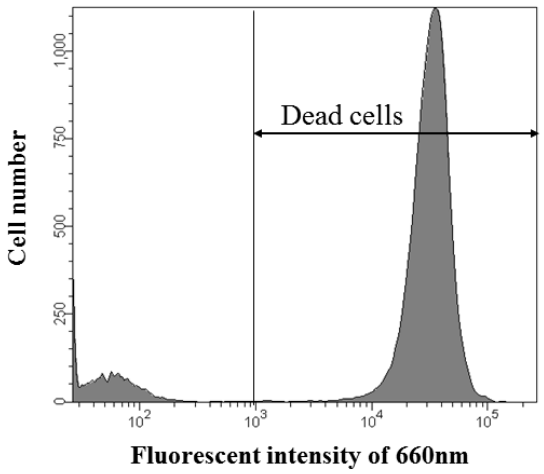

Fig. 7 Estimation of dead cells by flowcytometer

Table 1 Reproducibility of this new method

		Measurement number					Mean	Standard deviation	Valiation coefficient (%)
		1	2	3	4	5			
Viability	Dead cell ratio by TO-PRO3	4.1	4.0	3.8	3.9	3.7	3.9	0.16	4.1
	Dead cell ratio by Methylene Blue	2.5	3.0	2.7	4.4	3.1	3.1	0.74	24
Vitality	Mean	5.64	5.64	5.63	5.62	5.64	5.63	0.00682	0.121
	Median	5.79	5.79	5.79	5.78	5.79	5.79	0.00533	0.0922
	Standard	0.673	0.685	0.683	0.680	0.686	0.681	0.00460	0.675

section 2.4 above. These special dead cells were not stained with 5 (and 6)-carboxyfluorescein (data not shown). If this new method works, viability of these cells could be measured by TO-PRO3, with no information on vitality (intracellular pH) being detected.

Table 2 shows the results for viability (dead cell ratio) and vitality for the five samples that contained different proportions of dead cells prepared using the method in section 2.4.

As shown in table 2, yeast vitality (mean, median and standard deviation of intracellular pH) was similar among the five samples. However, yeast viability was measured based on the ratio of dead cells. This confirms that the present method is able to accurately and simultaneously evaluate viability and vitality.

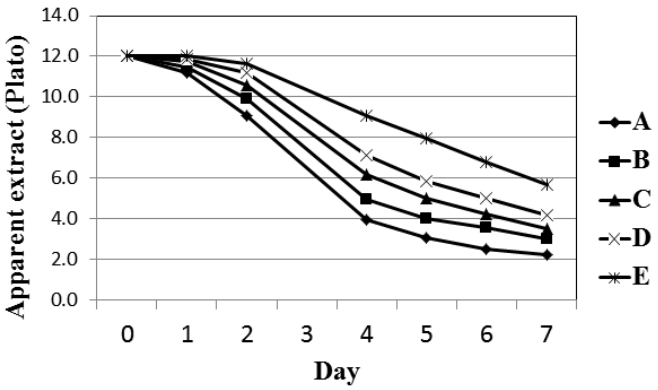

Fig. 8 Fermentation test of yeast samples in table 2

Figure 8 shows the results for fermentation test of these five samples. Fermentation performance of the five samples fluctuated, depending on yeast sample viability.

These results show that this new method is able to evaluate the physiological status of yeast samples with no correlation between viability and vitality. In addition, these results show that the measurement of either viability or vitality alone is not sufficient. To assess yeast physiological status precisely, we need to evaluate both viability and vitality simultaneously.

3.4 Investigation of correlation between viability and vitality in actual yeast samples

In order to confirm our assumption that there is not always a clear relationship between viability and vitality, 900 yeast samples obtained under actual brewing conditions were evaluated. The viability (dead cell ratio) and the vitality (mean of intracellular pH) of the 900 samples are plotted in figure 9. The correlation coefficient between viability and vitality was r = –0.368. Thus, there was no clear correlation between viability and vitality of actual yeast samples under actual brewing conditions; viability did not always fluctuate according to vitality and vitality did not always fluctuate according to viability. This result also indicates the fundamental problems with current methods for evaluation of viability and vitality; measurement of viability or vitality alone is not able to evaluate yeast physiological status under actual brewing circumstances.

As shown in figure 9 (see next page), when yeast vitality (mean of intracellular pH) was 6.2, there were the cases in which yeast viability (dead cell ratio) fluctuated markedly from 1.8% to 16%. If the intracellular pH is more than 6.0, the yeast cell is vital [15, 17]. When the yeast viability (dead cell ratio) was 5.6%, there were

Table 2 Simultaneous evaluation of both viability and vitality of prepared yeast samples

	Sample	A	B	C	D	E
	Additional rate of dead cells (*) (%)	0	25	50	70	90
Viability (TO-PRO3)	Dead cell ratio (%)	3	23	51	68	89
Vitality (intracellular pH)	Mean	5.42	5.40	5.39	5.37	5.24
	Median	5.55	5.53	5.52	5.51	5.45
	Standard deviation	0.772	0.767	0.783	0.826	1.02

(*) The dead cells were prepared by the method described in 2.4 of materials and methods

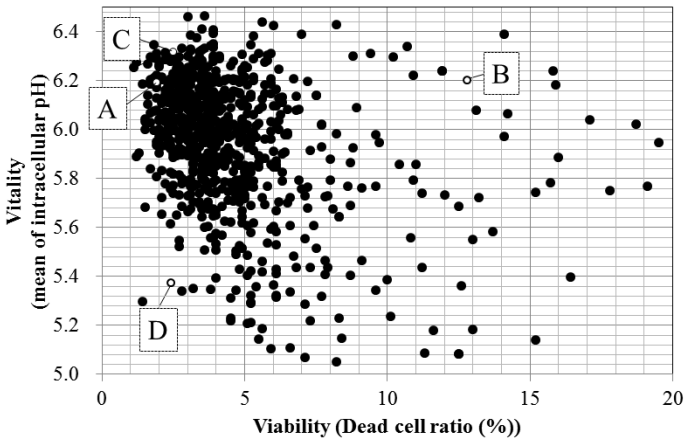

Fig. 9 Relationship between viability and vitality in actual brewing circumstances.

The different 900 samples in actual brewing circumstances were analyzed for three years. The viability (dead cell ratio) and the vitality (mean of the intracellular pH) were plotted. The correlation coefficient between viability and vitality was r= –0.368. There was not clear correlation between viability and vitality. A, B, C, D indicated yeast crop A, B, C, D in figure 10, 11

the cases in which yeast vitality fluctuated markedly from 5.2 to 6.4. Figure 9 clearly shows that it is necessary to simultaneously evaluate both viability and vitality in order to accurately assess the physiological status of yeast under actual brewing conditions.

Figure 10 shows an example of data obtained using the samples in figure 9. As shown in figure 10, the dead cell ratio fluctuated from 2.1 % to 9.6 %, although intracellular pH was similar (mean, median and standard deviation were similar). This might be caused by negative selection depending on yeast handling technology

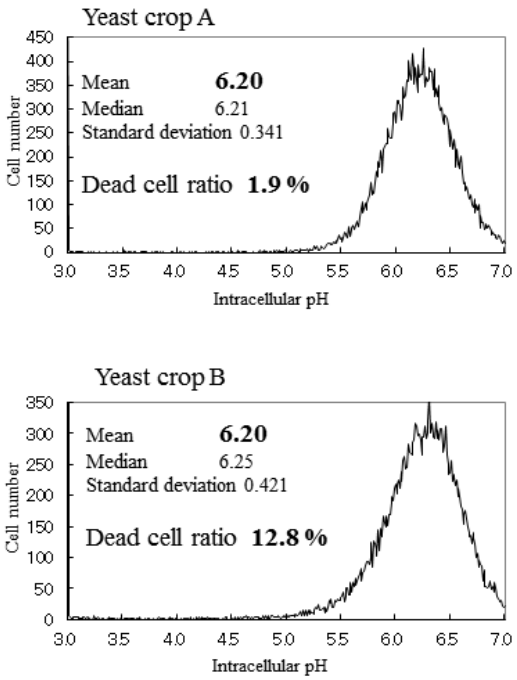

Fig. 10 Actual examples whose viabilities were different though vitalities were similar.

Yeast crop A, B were indicated in figure 9

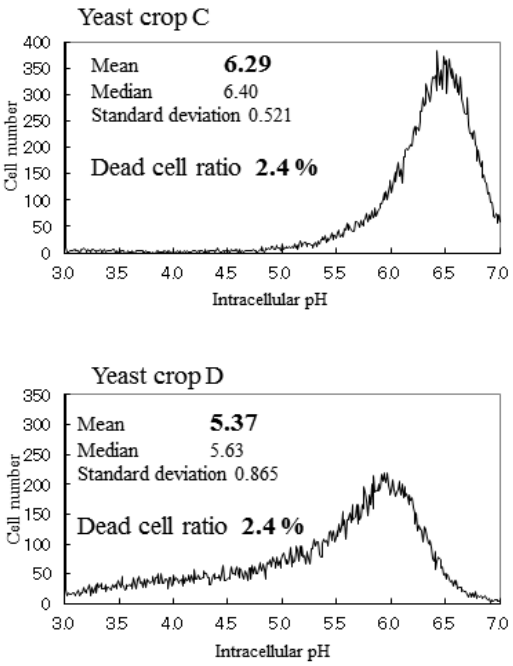

Fig. 11 Actual examples whose vitalities were different though viabilities were similar.

Yeast crop C, D were indicated in figure 9

(e.g. cropping procedure). Figure 11 shows another example from figure 9. The intracellular pH (vitality) fluctuated from 6.38 to 5.35, although dead cell ratio (viability) was similar. This might be caused by differences in brewing conditions, such as differences in wort composition or fermentation conditions. Originally, we have to evaluate yeast physiological conditions from the viewpoint of both viability and vitality. The method proposed in this paper could simultaneously evaluate both viability and vitality.

3.5 Fluctuations in viability and vitality of yeasts stored at different temperatures

In order to assess whether this new method evaluates subtle differences in yeast physiological status, fluctuations in the viability and vitality of yeasts stored under different low temperature conditions was investigated. Yeast suspensions stored at –2 °C were not frozen because yeast was suspended in beer and the suspensions contained 5 % ethanol. As shown in figure 12 (see next page), fluctuations in the distribution of intracellular pH and viability (ratio of dead cells) were observed using this new method.

3.6 Effects of both viability and vitality on fermentation performance and yeast metabolites

After yeast storage for 1 to 36 days at 0 °C, fermentation tests were performed using these stored yeasts. The same wort was used in this fermentation test. Fluctuations in yeast vitality distribution and viability during storage were plotted in figure 13 (see next page). Although the mean value of intracellular pH decreased during deterioration, the standard deviation increased from 1 to 4 in the sample, and the standard deviation fluctuated slightly when mean values were less than 5.0 (samples 5 to 6). Table 3 shows

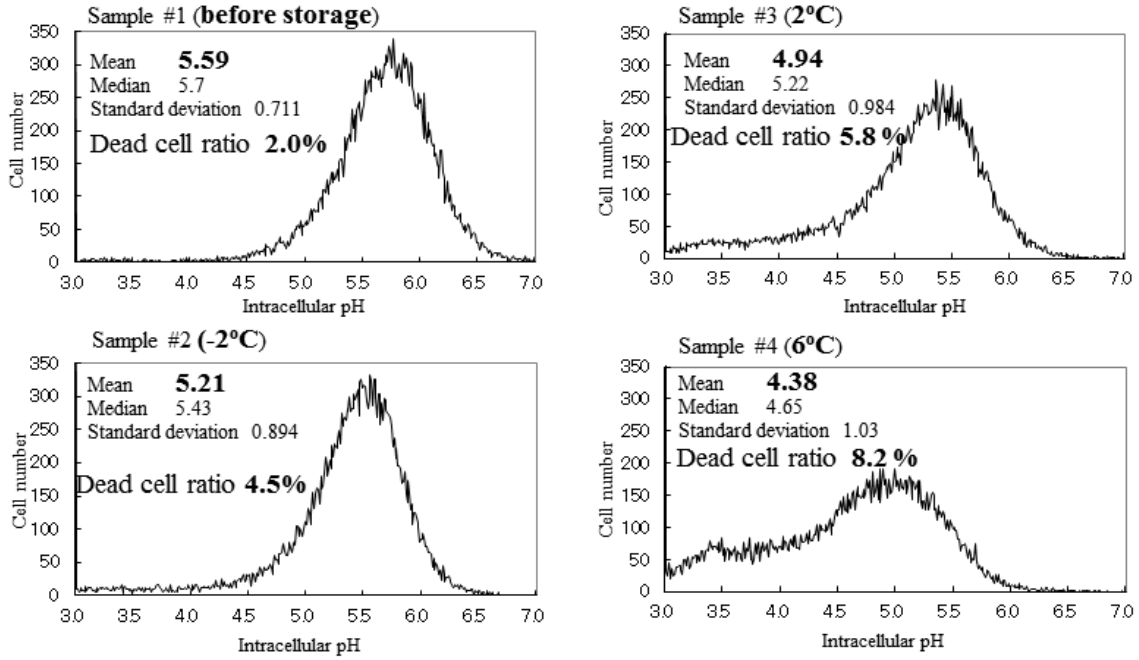

Fig. 12 Fluctuation of viability and vitality of yeast stored at different temperature.
The yeast samples were stored for 15 days at the indicated temperature in this figure

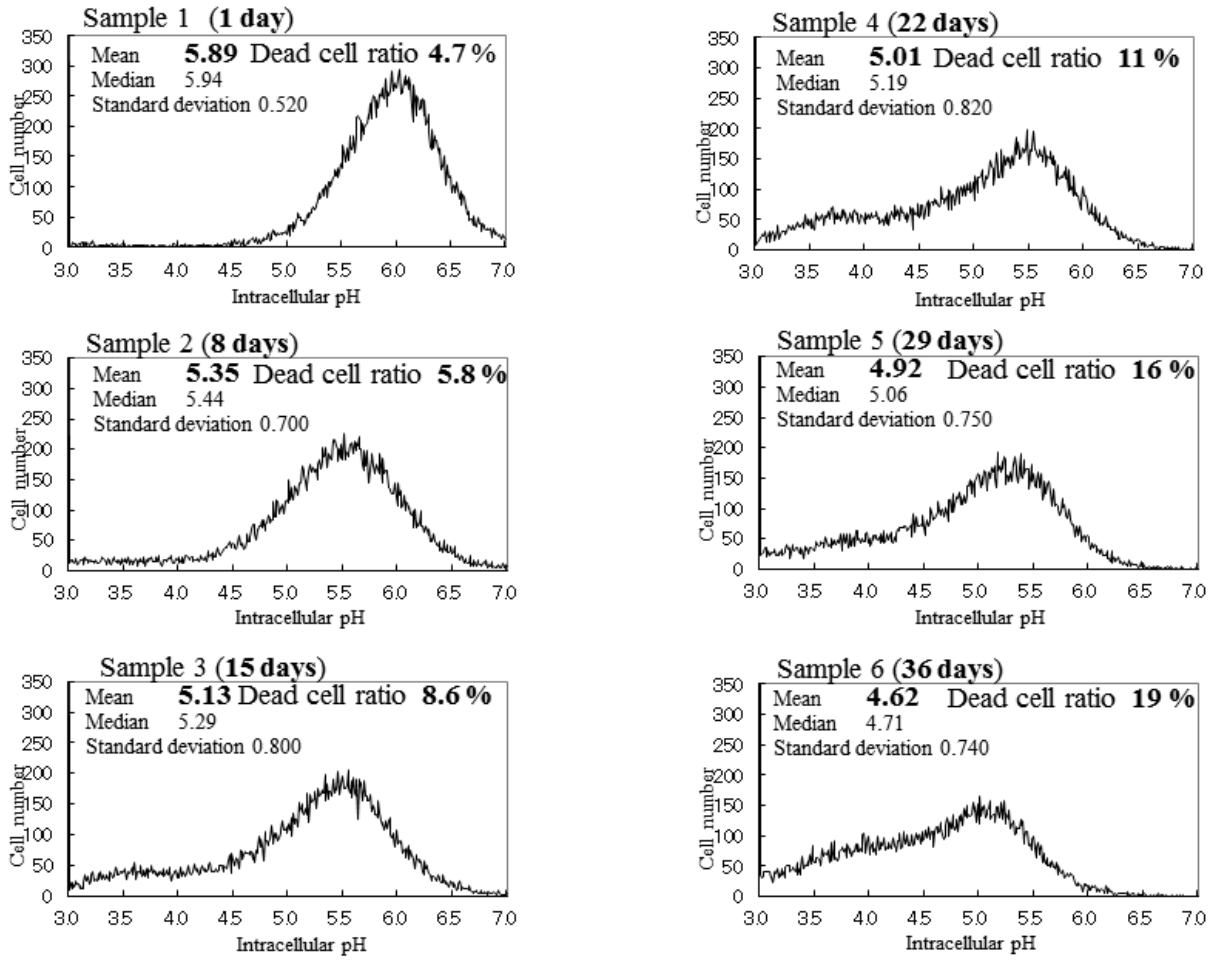

Fig. 13 Fluctuation of vitality and viability during yeast storage.
The yeast samples were stored at 0°C for the indicated days in this figure

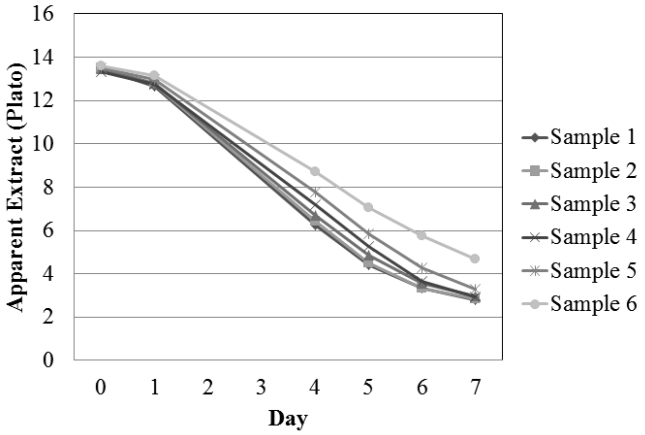

Fig. 14 Fermentation test of yeast samples in table 3

the pitched yeast vitality and viability, and the analysis of young beer after fermentation. Fermentation performance is shown in figure 14.

As shown in table 3, yeast vitality and viability decreased according to storage period. Though other than for sample 6, similar results were obtained for fermentation performances as shown in figure 14, analyzed compounds (pH, esters, diacetyl, amino acid consumption, middle chain fatty acids, H_2S and SO_2) (Table 3) fluctuated according to yeast viability and vitality. In particular, amino acid consumption, ethyl caprylate, caprylic acid, capric acid, H_2S and SO_2 fluctuated markedly in the case of samples 1 and 2, whose viability (dead cell ratio) fluctuated little (4.7 to 5.8), but whose vitality fluctuated very much (5.65 to 5.35). Even if viability fluctuated slightly, when vitality fluctuated in the range of less than 5.65, yeast metabolites fluctuated. It was also remarkable that iso-amylacetate, total diacetyl, amino acid consumption, caprylic acid, capric acid, H_2S and SO_2 fluctuated markedly in the case of samples 3, 4 and 5, whose vitality did not fluctuate substantially (5.13 to 4.92), but whose viability fluctuated very much (8.6 to 16 %). Even if vitality fluctuated slightly, when dead cell ratio fluctuated in the range of over 5 %, yeast metabolites fluctuated. These results demonstrate the need to evaluate both vitality and viability simultaneously. The present method is able to simultaneously evaluate both viability and vitality under actual brewing conditions, and to estimate the quality of beer brewed by the assessed yeast cells.

4 Conclusions

In order to assess the physiological status of yeast, either viability or vitality alone has been measured to date. However, in many cases, there was no correlation between viability and vitality under actual brewing conditions. To resolve this fundamental problem, the simultaneous evaluation of both viability and vitality was described in this paper. By applying the ICP method and the TO-PRO3 method, we were able to accurately evaluate both the viability and vitality of individual cells simultaneously. This study demonstrated the reproducibility of this new method and its suitability for assessing yeast samples obtained under actual brewing conditions, in which there was no correlation between viability and vitality. This method was also able to predict subtle differences in fermentation performance and yeast metabolites. In addition, 30,000 individual cells could be evaluated in a few minutes using a flow cytometer. As this method was able to simultaneously evaluate viability and vitality, the fundamental problems of the current evaluation methods for viability and vitality were resolved. The concept of evaluating both viability and vitality simultaneously can contribute to the brewing of quality beer.

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Table 3 Yeast physiogical status and yeast metabolites after fermentation

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Storage period (day)	1	8	15	22	29	36
Vitality (Mean of Intracellular pH)	5.65	5.35	5.13	5.01	4.92	4.62
Dead cell ratio (TO-PRO3)	4.7	5.8	8.6	11	16	19
Analytical results of young beer						
Apparent Extract (%)	2.73	2.75	2.90	2.86	3.25	4.64
pH	4.36	4.37	4.39	4.39	4.43	4.54
iso-amylacetate (mg/l)	2.0	2.2	2.3	2.1	2.8	2.9
Total diacetyl (mg/l)	0.34	0.35	0.35	0.44	0.46	0.42
Amino acids consumption (mg/l)	1362	1220	1326	1216	1091	1126
Ethyl caprylate (mg/l)	0.06	0.09	0.09	0.11	0.10	0.18
Caprylic acid (mg/l)	1.19	1.31	1.37	1.39	1.50	1.66
Capric acid (mg/l)	2.87	3.12	3.37	3.37	3.67	3.8
H_2S (mg/l)	10.4	16.4	20.6	20.5	31.8	36.8
SO_2 (mg/l)	3.14	4.02	4.28	4.74	5.42	8.12

Gray columns show fluctiation of vitality, dead cell ratio and yeast metabolites

5 Literature

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