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Studies on Influence of Hydrostatic Pressure on Yeast Cells and Application to Industrial Yeast for Developing New Methods

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(高圧が酵母細胞に与える影響と産業用酵母の新しい
育種方法についての研究)

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1. INTRODUCTION

1.1 Histories of application for high pressure on physical, chemical and biological science.

In the late nineteenth century, in the Talisman expedition, research was developed in various directions as a result of the existence of microorganisms in the deep sea about 5000m by A. Certes, C. R.(1984). In particular the mechanism of these responses had been studied using deep-sea microorganisms. In the case of bacteria, growth of bacterial mycelium promotion, spore germination (Y. Horie 1991), promotion of sporulation (G. R Bender 1982) were good examples. Inside the cell, cell division which was thought to be caused by DNA replication inhibition and damage of the cell division mechanism delayed and inhibition phenomenon also appeared, but the mechanism of cell response to high pressure stress had not been fully clear.

Although there had been many cases of studying the phenomenon of change caused by high pressure in physical and chemical aspect in many cases from the 1990 's. Regarding the mechanism of the response to high pressure stress which was focused deep sea microorganisms, how the function of bacterial cell walls and cell membranes, which will directly receive this stress, were maintained, especially the phospholipids (Y. Horie 1991), (K. Kamimura1993).

For example, the amount of unsaturated fatty acids in cell membranes of hypertensive bacteria increased according to increase high pressure stress, by increasing pigment of unsaturated fatty acid, it kept cell membrane's fluidity and could grow by maintaining material permeability function.

Furthermore, research had also been made to elucidate the active side of several enzymes

which were responsible for substance permeations and energy metabolism systems present in cell membrane. *Phosphoenolpyruvate carboxyl phosphotransferase*, which is one of the enzymes involved in sugar transport system, maintained its function even under high pressure and activity of *NADH oxidase* could be maintained even under high pressure (C. E. Zobell 1970) . The mechanism of response held by deep-sea microorganisms against high pressure was extremely complicated.

Recently, researches had been started on the mechanism of regulation of gene expression in response to high pressure stress, and genes that were specifically expressed under high pressure had also been isolated considering effective utilization of genetic resources of deep-sea microorganisms (Y. Horie 1991), (K. Kimura 1994) .

1. 2 Effect of high pressure on microorganism

In comparison to pressure levels that cause growth inhibition, cellular death occur in relatively larger pressure range depending on species of microbes. *Escherichia. coli* (Sonoike K. 1993) is dead under 550 MPa. , but eukaryotic microorganisms such as yeast are more sensitive to high pressure. *S. cerevisiae* can be inactivated under around 40-150 MPa (Iwahashi H. 1991).

The crucial damage that cause cellular death by pressure is only explained that heat shock treatment induced bar tolerance using *S. cerevisiae* (Iwahashi H. 1991). The heat shock treatment on cells are accumulated Hsps and trehalose, Hsps plays the role to repair and break down proteins and trehalose stabilizes proteins and membrane structures (Iwahashi H 1995). However, the consideration is doubtful because proteins are damaged under much higher pressure conditions (Iwahashi H. 2015).

High pressure was damaged to yeast cells due to leakage of several internal substances such as several kind of amino acids, minerals and glutathione from the cells (Y. Horie 1991). Leakage of contents from inside of the cells were presumed to be due to destruction of the cells and cell wall by high pressure. As part of research on response mechanism of microorganisms against high pressure stress, a mechanism of damage and occupation of the cells which were one of cellular responses by high pressure stress using *S. cerevisiae, Candida tropicalis* and *S. pombe*.

The phenomenon induced by cell hyperplasia induced by high pressure stress, which was clarified in the results of the previous research, was a phenomenon very similar to the cell multiplication seen in the treatment of microtubules inhibitors. This result suggested that high pressure stress had a significant influence on cell fission devices and cytoskeleton.

High pressure has dramatic effect on the cytokinetic and mitotic activities of microorganisms (Zimmerman, AH. 1971). Pressure above 100MPa, microorganisms were rapidly dead because

of internal structure damage (ZoBell, CE. 1970). Deep sea microorganisms originally had function capable of growth under high pressure. Several numbers of reports on the influence of high pressure in microorganisms have been investigated (Schmid G 1975), (Thibault J 1987), but there were few studies of yeast cells effected by high pressure were generally growing under normal pressure.

In general, hydrostatic pressure has dramatic effects on cytokinesis and mitotic activities of dividing cells (Zimmerman, AH 1971). It especially inhibited replication of DNA in bacteria (Landau JV 1970), marine eggs (Zimmerman AM. 1967), and mammalian cells. (Landau JV 1970) In addition, hydrostatic pressure treatment is widely used to duplicate the chromosome sets of fishes (R. K., Mortimer 1996)

OMICS technology shed light on the damages on cellular organelles during high pressure conditions. Transcriptome analysis of yeast cells that repairing crucial cellular damage suggested a lot of genes whose products were localized in the endoplasmic reticulum, mitochondria and nucleus were highly activated (Iwahashi H 2003).

This phenomenon suggested the crucial damages were in cellular organelles. The transcriptome analysis found 286 activated genes during recovery conditions. In these genes 13 genes were essential genes to alive for yeast cells and almost all genes contributed on proteasome functions. Thus, proteasome seemed to be essential for yeast cells to recover the functions after high pressure (Tanaka Y 2010).

The metabolomics suggested the significant accumulation of glycine, valine, isoleucine, leucine, asparagine, aspartic acid, and tyrosine in the cells (Tanaka Y 2010). Among these amino acids, glycine, valine, isoleucine, leucine, and tyrosine are known to be dominant in the membrane-spanning domain of transmembrane proteins. The metabolomics analysis agrees with genomic analysis that membrane structures including membrane protein can be the critical event that cause yeast death. Membrane structures are shared by cellular organelles and there must be

crucial damages in cellular organelles.

1. 3 Application of developing new type of yeast strains

For developing yeast strains, it has been still common to use method by ordinary sexual joining and rare mating until today. Regardless of which methods has been used, it is essential to search useful properties from the original strains or to generate useful properties from the original strain by mutation treatment. The mutation of yeast cells are used chemical treatment such as EMS, NTG, Benomyl etc, and physical treatment such as UV, cobalt irradiation, heat, and drying have been conventionally used.

To analyze random samples of liberated ascospores, several procedures have been used in *S. cerevisiae* (R.K., Mortimer 1969). The ascus wall has to be disrupted mechanically (C. C. Emeis 1958) or dissolved enzymatically (J. R. Johnston 1959). Obtaining a random spore sample, two problems are encountered. One is the separation of the ascospores from vegetative cells, and another is micromanipulator which is laborious and time-consuming to less experienced workers.

The difficulty of these problem was partially overcome by treating vegetative cells with glass beads (C. C Emeis 1958), ultrasound (G. E. Magni 1963), ultrasonic paraffin (B. A. Siddiqi 1971), ethanol (L. A. Azkharov 1964) and ether (I. W. Dawes 1974). Until now, pressure treatment had not been proposed for selecting ascospores from populations containing vegetative cells.

1．4　Purpose of this study

There are a lot of interesting phenomenon by high pressure on prokaryotic microorganisms, such as yeast and plant, but almost all of these phenomenon are investigated on laboratory strains. And there is little knowledge about *S. pombe* which is close to mammalian cells. We have studied the influence on industrial baker's yeast, *S. cerevisiae* and laboratory strains *S. pombe* by high pressure.

It had been found that these strains were affected by high pressure as known in other strains and died up to 100MPa or more. The cells of exponential growth phase were treated by high pressure, we could obtain several colonies with different colors onto the dye agar plate, and some of these cells had increased ploidy. In order to clarify which organelle in the cells were affected by high pressure, and investigated why polyploid cell could be obtained, and also to clarify whether high pressure treatment is useful as one of the methods for improving industrial yeast strains.

Furthermore, using laboratory strains *S. cerevisiae,* we investigated which part of the cell is susceptible to pressure by using a fluorescence microscope. We verified whether hydrostatic pressure is one of effective method for improving new kind of industrial yeast strains.

2. MATERIALS AND METHODS

2.1 Strains and culture conditions

Industrial strains, *S. cerevisiae* were all from our laboratory collections. The strain *O-39* (diploid *a/α*) was commercially as a baker's yeast. The strains *P-544* (diploid *a/α*) have been deposited at the Fermentation Research Institute, Tsukuba, Japan, and are available as *S. cerevisiae P-11426* and *S. cerevisiae P-1153*0, respectively. The haploid strain *HF399s1* (*MAT α*) and *Ha112* (*MAT a*) was used in crosses.

Laboratory strains, *S. cerevisiae BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0)* was for the control strain and its drivaties of GFP labeled constructs (Hun W 2003) and the strain containing *p*PTR2-4K＞R-GFP were employed (Table). The plasmid *p*PTR2-4K＞R-GFP contains GFP-tagged *Ptr2* proteins constructed and kindly provided by Fumiyoshi Abe (Kawai K 2014). The laboratory strains labelled by GFP used in this study showed at Table1.

Table 1 GFP labelled yeast strains

Labeled Organelle	Strain	Genotype	Ref.
	S. cerevisiae BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	
Nucleus membrane	*S. cerevisiae* BY4741 GFP-Nic96 YFR002W	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 NIC96:GFP (S56T)-HIS3MX6*	8
Nucleus	*S. cerevisiae* BY4741 GFP-Rox3 YBL093C	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ROX3:GFP(S56T)-HIS3MX6*	8
Endoplasmic Reticulum	*S. cerevisiae* BY4741 GFP-Pho86 YJL117W	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PHO86:GFP(S56T)-HIS3MX6*	8
Golgi Body	*S. cerevisiae* BY4741 GFP-Sec7 YDR170C	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SEC7:GFP(S56T)-HIS3MX6*	8
Cellular membrane	*S. cerevisiae* BY4741 pPTR2-4K > R-GFP	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* *p*PTR2-4K > R-GFP	9

The fission yeast *S. pombe* strains *JY1* (*L972*h^{-}), *JY3* (*L975*h^{90}), *JY333* (*ade6-M216 leu1*h^{-}), *JY334* (*ade6-M216 leu1* h^{+}) and *JY276* (*his2/his2 ade-6-M216/ade6-M210* h^{-}/h^{+}) were kindly provided by Prof. M. Yamamoto, University of Tokyo, Japan, and the variants *JY1V1, JY1V2, JY1V3* described in this work were derived from pressure stress of *JY1* (wild type, *L972*h^{-}).The basic medium for culture growth was YPD medium containing 2% poly peptone, 1% yeast extract and 1% glucose. For the solid medium, agar was added at final concentration of 2%. If needed adenine (100mg/L) was added in YPD medium.

Yeast cells were grown in YPD medium at 30°C with shaking. For visualized structures of organelles after pressure treatment study were carried out according below. Pre-cultures of *S. cerevisiae* were grown in YPD (2% poly peptone, 1% yeast extract, and 2% glucose) at 25◦C for 2-3 days. The pre-culture was diluted and grown overnight to optical density (OD 660 nm) of 1.0 (Iwahashi H 2003), and the colony-forming units (CFU) were estimated on YPD agar plate.

The scale up test of industrial strains were carried out below. Starter cultures grown in test tubes, subsequent pre-cultures grown at reciprocal shaker containing 100 ml medium and incubated at 30°C for 48 hours. The medium composition was contained (per liter): 10g $(NH_4)_2SO_4$, 1g KH_2PO_4 and 100g clarified cane molasses. Clarified cane molasses was the sole

carbon and energy source, and had a sugar content expressed as a glucose equivalent of 330 g/L. We initially added 1.0 L growth medium, tap water and nitrogen source to 30L jar fermenter, so that the final volume reached 16 L after molasses and phosphoric acid were added successively. All fermentations were performed at 30°C. The final pH of the medium was adjusted to 5.5 unless otherwise specified.

For fed-batch cultivation, molasses was added successively with a peristaltic pump under either manual or computer control. After cultivation, yeast cells were harvested, washed three times in distilled water and used immediately. In use, the cells were suspended in water or 0.1 M phosphate buffer, pH 7.0 to final concentration of 100 mg wet weight per ml.

For pressure stress experiments of *S. pombe*, cells were grown to the stationary phase in YPD medium on a reciprocal shaker at 30°C.

2-2 Pressure treatment

Cell suspensions (10% w/v) were used to fill a 5ml collapsible polyethylene bottle, which was tightly stopped at one end. This was immersed in kerosene or light paraffin in a sample compartment of pressure pomb and compressed to 800 MPa with a hand-type oil pressure apparatus (Type KP-10B, Hikari Koatsu Co., Hiroshima). The pressure apparatus was enclosed in a temperature-controlled housing at 25°C.

The laboratory strains labelled by GFP used in this study were used exponentially growing yeast cells subjected to a various degrees of pressure conditions for 16h at 0℃. After decompression, yeast cells were observed by fluorescent microscope with exitation of 490 nm and emmision of 510 nm.

2-3 Viability measurements

Following decompression, culture samples were taken under aseptic conditions, diluted with 0.85% NaCl and plated on YPD agar plates. These were incubated at 30°C for 2 to 4 days prior to counting colonies. The cell survival of a pressure treated culture was calculated as below. (colonies forming unit after pressure) / (colonies forming unit before pressure)

2-4 Measurement of cell number and size

The increase in cell number and size were calculated, using an Elzone particle counter 80XY (Particle Data Inc.) which was equipped with 48 μm orifice tube and a 100 μl volumetric tube. The current and gain were at 4.0 and 1.0, respectively. For the haploid strain *HF399s1* and its hybrids, the mean value for cell size was calculated from direct measurement of 50-100 cells on a light microscope/Image Analyzer SPICCA II Image Command 5098 (Nippon Avionics Co., Ltd.), on the assumption that cells spheres.

2-5 Determination of cell ploidy

The determination of the degree of ploidy in yeast cells was performed exactly as described by Aigle et al (Aigle M 1983). This involved measuring the DNA contents per cell of the strains, by using the particle counter and determining the amount of DNA by the diphenylamine colorimetric reaction.

2-6 Induction and detection of variants

Cells were grown in YPD medium to late log phase or stationary phase, centrifuged, and washed twice with 0.85% NaCl. The cells were re-suspended in 5 ml of the same solution and a concentration of approximately 1×10^9 cells per ml, and pressured at 100-300 MPa for 10 min at 25°C. After pressure treatment, culture samples were immediately taken under aseptic conditions, diluted with saline, spread on a dye plate containing the following per liter: 11.7 g of yeast carbon base (Difco Detroit. Mich.), 40 g of sucrose, 3 g of yeast extract, 5 g of methionine, 25 mg of aniline blue, 50 mg of Ponceau 3 R, and 20g of agar and incubated at 25°C for 4 - 5 days.

The colony color of variants onto the plate turned from violet to red/blue or dark blue. In contrast, the colony color of untreated by high pressure and parent colony remained violet.

2-7 Genetic analysis for *Saccharomyces cerevisiae* and *Shizosaccharomyces pombe*

Random spore isolation and mass-mating were performed according to standard procedures. (Nakatomi Y 1988) In brief, for random spore isolation, ascus suspensions were treated with zymolyase (Seikagaku kogyo, Japan) followed by a gentle grinding with powdered glass to destroy the vegetative cells and be free ascospores. The resulting spore suspensions were spread onto YPD agar plate for 2 days for germination and growth of spores. To measure the sporogenesis rate, cells cultivated on sporulation culture medium was examined with microscope, and 200-500 cells or asci were counted per sample. On the other hands, standard *S. pombe* genetic procedures were used for genetic crosses, sporulation, and random spore analysis. (Moreno, S. 1991) Cells from strains to be tested were incubated 2 days at 30°C on supplemented YPD medium containing 100mg/L adenine (YPDS).

Mating mixtures were created by mixing two strains with opposite mating type on MEL agar plates (3% malt extract, 0.68% KH_2PO_4, 100mg/L adenine-HCl, 2% sugar), followed by incubation for 3 days. For random spore analysis a heavy loop of sporulated cultures was

transferred to a solution of 30% ethanol and incubated 40 min at room temperatures to kill the vegetative cells.

Then the spores were spread on YPDS plates. The resultant colonies were replica-plated from YPD to SD (0.17% Yeast Nitrogen Base without amino acids, 0.5% $(NH_4)_2SO_4$, 2% glucose, 2% agar) plates supplemented with nutrients (adenine and leucine).

2-8 Chemical analysis

After pressurization, 10% (w/v) cell suspensions were centrifuged and the cells discarded. In the supernatants, ultraviolet (UV)-absorbing substances were determined at 260 or 280 nm in a Shimazu 160 spectrophotometer. The mineral content of supernatants was determined by atomic absorption equipment, 5 ml portions of the supernatants were transferred to glass tube and acidified with an equal volume of HNO_3 and $HClO_4$ treated at 200°C for 1 h.

The amino acid content of supernatants was determined by a Hitachi L-8500 amino acid analyzer. Glutathione released from the pressure-treated cells was determined by an enzymatic methods based on *glyoxalase I* (Bernt E. 1974).

3 Results

3-1 Morphological changes in yeast cells of *S. cerevisiae* induced by hydrostatic pressure

S. Shimada et al (S. Shimada 1993) investigated the morphological change of yeast due to high pressure and substances leaked from yeast cells using industrial baker's yeast strains. Utilizing SEM, the outer shape of the cells when treated with pressure up to 300 MPa at room temperature (Fig. 1b and 1c), and looked the same as untreated cells (Fig. 1a), but at pressure higher than 500 MPa there was disruption (Fig. 1d), or damaged in the area of bud scar of the cell wall (Fig. 1e), which were observed on 20-30% of the cell. TEM (Transmission electron microscope) revealed that the inner structure of the cells treated with hydrostatic pressure began to decompose, especially the nuclear membrane, even at 100 MPa (Fig. 2b).

The outer shape of cell at 300MPa was almost unaffected. But more than 500Mpa. 20-30% cells was disruption and damaged in the area of the bud scar of the cell wall. With increasing pressure above 400MPa cells were strikingly damaged and most of the intracellular organelles such as nucleus, mitochondria, endoplasmic reticulum, and vacuole were disrupted. At more than

400-600Mpa, further alternations appeared in the mitochondria and cytoplasm (Fig. 2).

They also tested the effect of hydrostatic pressure at subzero temperature (Fig. 2b). Pressure treatment at -20°C for 3h resulted in more severe damage to the inner structure of cells even at 200 MPa, although such alternations in the inner structure of cells were not observed when cells were exposed to increasing durations of up to 3h with pressure of 200 MPa at room temperature (data not shown). Almost all of the nuclear membrane were disappeared.

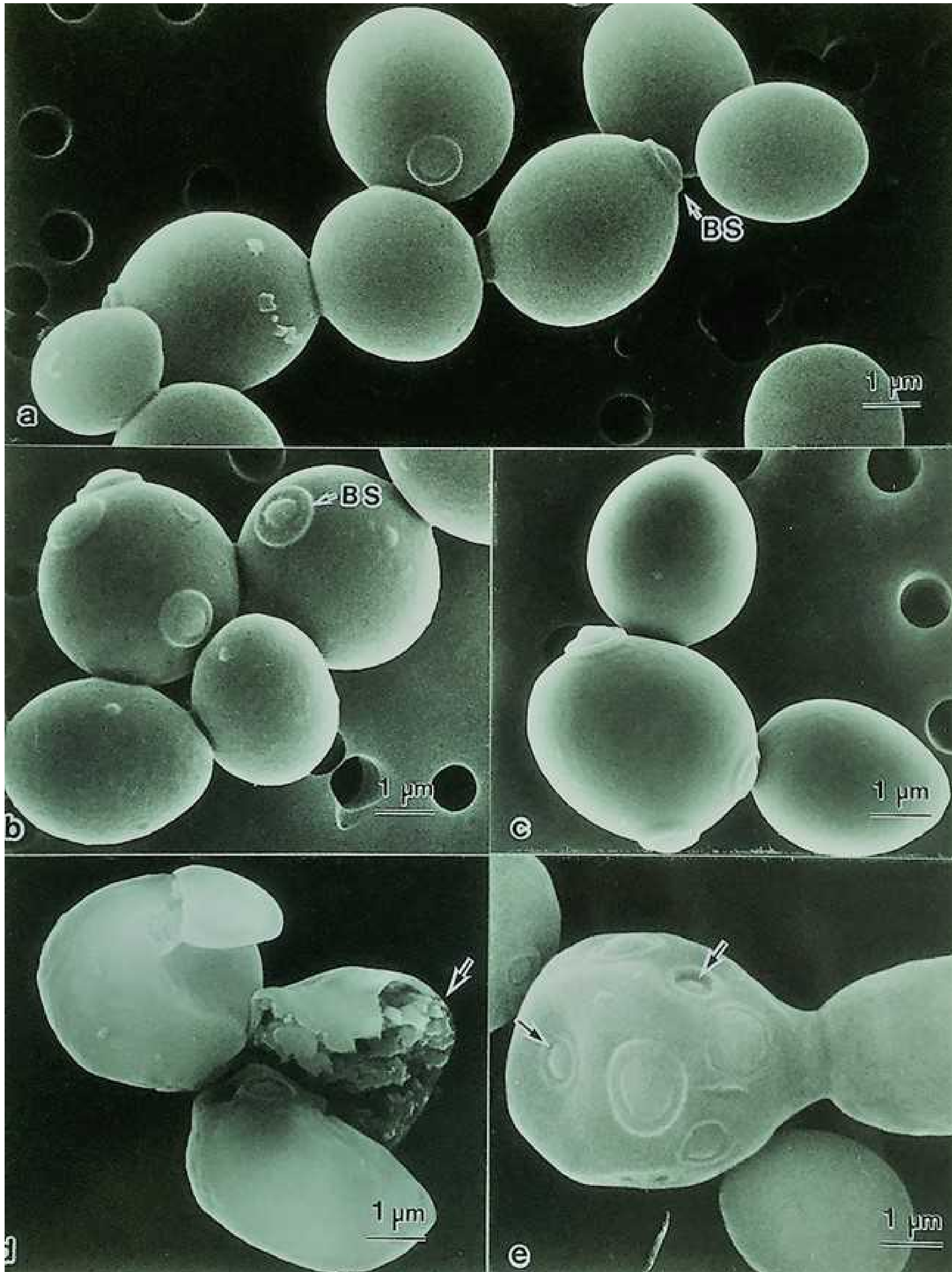

Fig. 1 a-e Scanning electron microscopy (SEM) image of *S. cerevisiae* O-39 cells treated without (a) and with hydrostatic pressure at 100 MPa (b), 300MPa (c), 500MPa (d), and 600MPa (e) for 10 min at 25°C: BS, bud scar (S. Shimada et al. 1993).

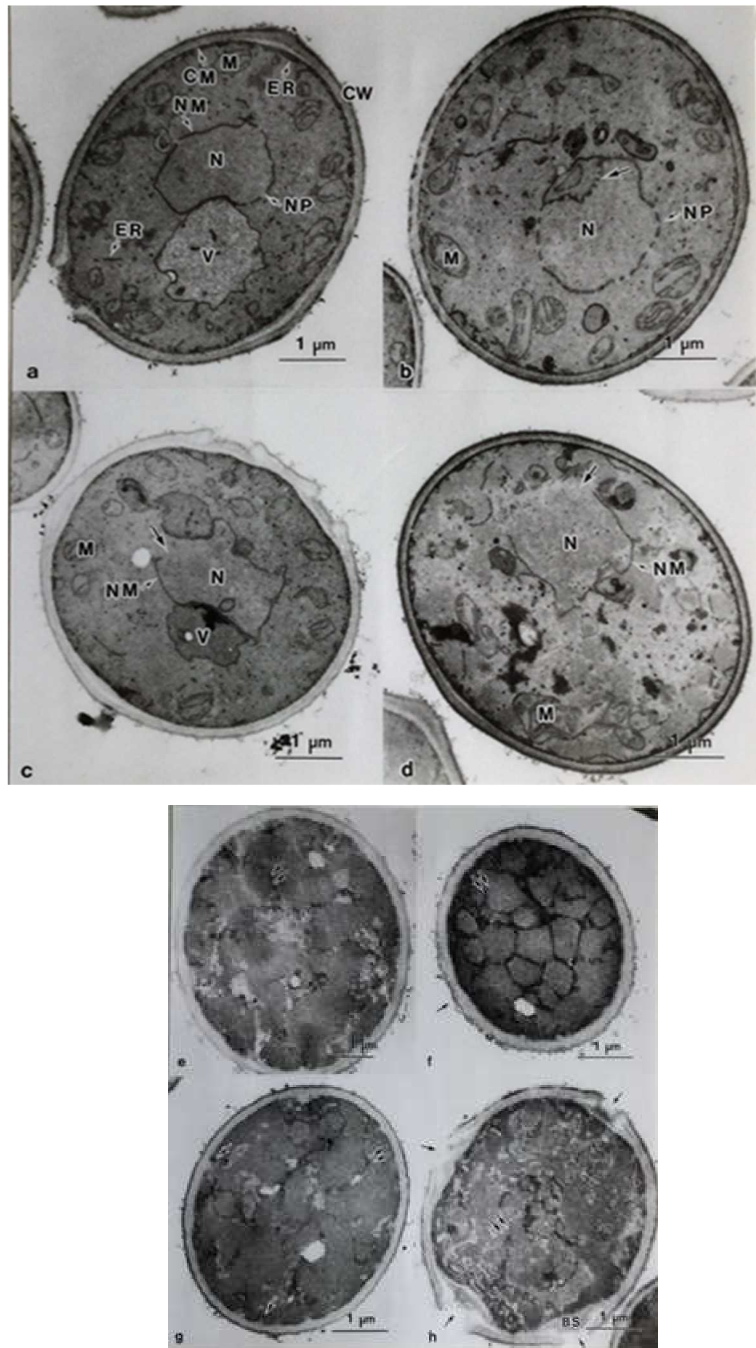

Fig. 2 Transmission electron microscopy (TEM) images of *S. cerevisiae O-39* cells treated without (a) and with hydrostatic pressure at 100MPa. (b), 300MPa. (c), 400MPa. (d and e), 500MPa. (f), and 600MPa. (g and h) for 10 min. at 25℃ (S. Shimada et al. 1993). CM: cell membrane, CM1: invagination of cell membrane, CW: cell wall, ER: endoplasmic reticulum, M: mitochondria, N: nucleus, NM: nuclear membrane, Np: nuclear membrane pore, V: vacuole

Table 2 Effect on the yeast nuclear membrane subjected to various pressures for constant duration of 10 min at 25°C or 200 MPa -20°C for 3h (S. Shimada et al. 1993).

Pressure conditions	Structure of nuclear membrane type[a]					Total cell no.	Abnormality (%)
	1	2	3	4	5		
0.1 Mpa	40	0	0	0	0	40	0
100 Mpa	37	6	0	0	0	43	14
200Mpa	13	20	7	7	0	47	73
300Mpa	11	15	11	12	10	59	81
400Mpa	4	16	2	8	14	44	91
500Mpa	0	8	1	14	8	31	100
600Mpa	0	0	0	0	40	40	100
0.1Mpa, -20℃	50	0	0	0	0	50	0
200Mpa, -20℃	0	0	0	0	50	50	100

* Type 1: nomally present, 2: abnomally present, 3: open type, 4: fragmentation, 5: absent

Under high magnification, nuclear membrane morphologies of pressure treated cells fell into four categories of abnormality in relation to the nuclear membrane pore size (Table 2). The percentage of abnormal structure in the nuclear membrane from these cells were 14, 73, 81 and 91 at 100, 200, 300 and 400 MPa, respectively, and 500 MPa the nuclear was not observed at all. These observation suggested that structural impact of high pressure on yeast cells occurred directly on the membrane system, particularly the nuclear membrane.

Even at relatively low pressure of 100-200MPa, the nuclear membrane showed a large number of nuclear pores, partially abnormal nuclear membranes, and sites where nuclear steric structure was not normal. At 300MPa or more, the nuclear morphology did not show the original circular shape, the nuclear pore became large, and destroyed mitochondrial outer membrane was also observed. The above results showed that the impairment of yeast to prominent cells under high pressure was in the nuclear membrane, and nearly 100% of the nuclei are affected at 500MPa or more.

Such phenomenon was not limited to only yeast cells. In fact, even in animal cells, there had been cases in which membrane protein chambers were successfully separated efficiently by high

pressure stress.

Furthermore, when high pressure treatment was carried out at -20°C, the inner structure of the cells were severely damaged even at 200MPa, and almost all of the nuclear membrane disappeared. The fluorescent nucleus in the cytoplasm was visible by 4, 6-diamidino -2-phenylindole (DAPI) staining. High pressure on the budding yeast *S. cerevisiae*, and the dimorphic yeast *C. tropicalis* revealed that membrane systems, especially the nuclear membrane were most susceptible to pressure stress even at 100MPa (S. Shimada 1993) , (Emis VCC 1960).

Hyphal cells were found to be more sensitive to pressure treatment than yeast form cells and microtubules to be more sensitive than microfilaments. The influence of high pressure on yeast cells was observed using an electron microscope. Compared to the cells grown at 0.1MPa (normal temperature), the cells subjected to high pressure treatment at 100-300MPa showed little change in the external shape. However, when subjected to high pressure treatment at 500MPa, the cells deformed, broken cells appeared, the cell morphology was deformed at 600MPa, irregularities appeared on the cell surface layer, and many cells with depressed spontaneous marks were observed. The internal structure of the cell also showed little influence up to 300MPa, but the cell structure rapidly changed at 400MPa or more, the intracellular organelle was not observed at all and a fragment thought to have destroyed the organelle was observed (Fig. 2).

3.2 The crucial damage that cause cellular death of *S. cerevisiae* by high pressure

To clarify the effect of pressure to yeast survivals (K. Hamada 2018), we estimated CFU after pressure treatment (Fig.3) as the yeast strains were genetically modified and they may have their own pressure resistances. Percent values were relative to CFU without pressure treatment.

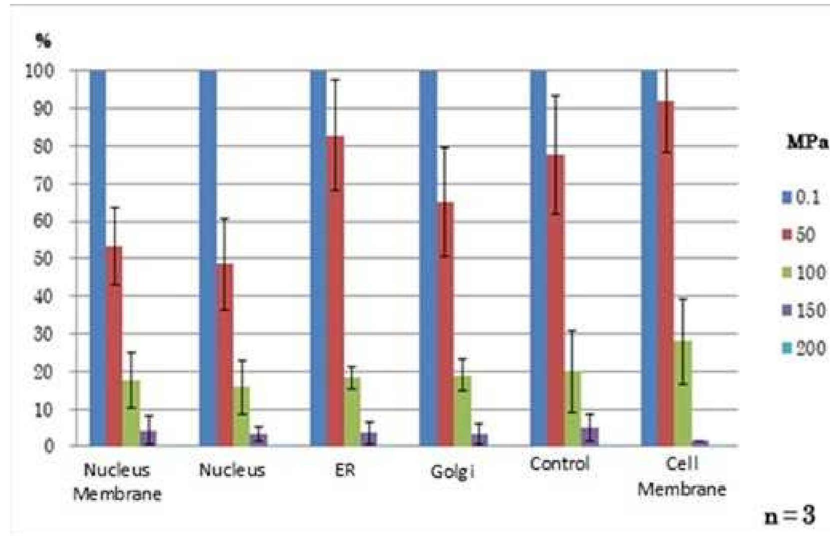

Fig. 3. Cell viability after pressure treatment under pressure range from 50 to 200MPa at 0 °C. GFP labeled organelles was shown instead of strain name.

It is true that each strain has original pressure resistance, however, there are significant decreases in CFU after 50MPa pressure treatment and majority of yeast cells were dead after 100MPa pressure treatment with all the strains. Thus, there must be critical damages in organelle structures up to 100MPa, on the other word, if cellular organelles are stable after 100 MPa those organelles are not critical (K. Hamada 2018). To visualize more clearly, we fused images among microscopic and fluorescent microscopic images. The example of cellular membrane labeled microscopic image, fluorescent microscopic image, and fused images are shown in Fig.4.

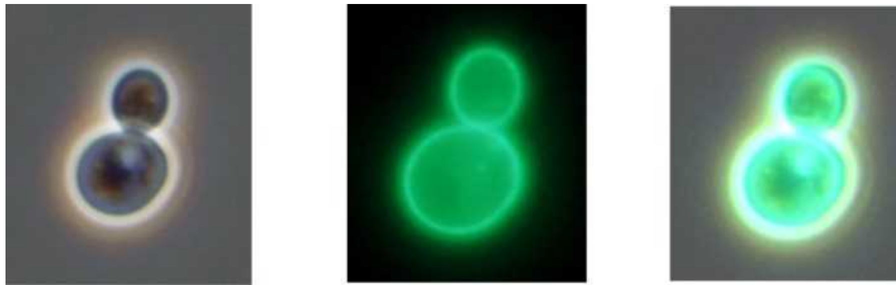

Fig. 4 Fusion of images among microscopic and fluorescent microscopic images.

A; microscopic image, B; fluorescent microscopic image, C; Emerged image.

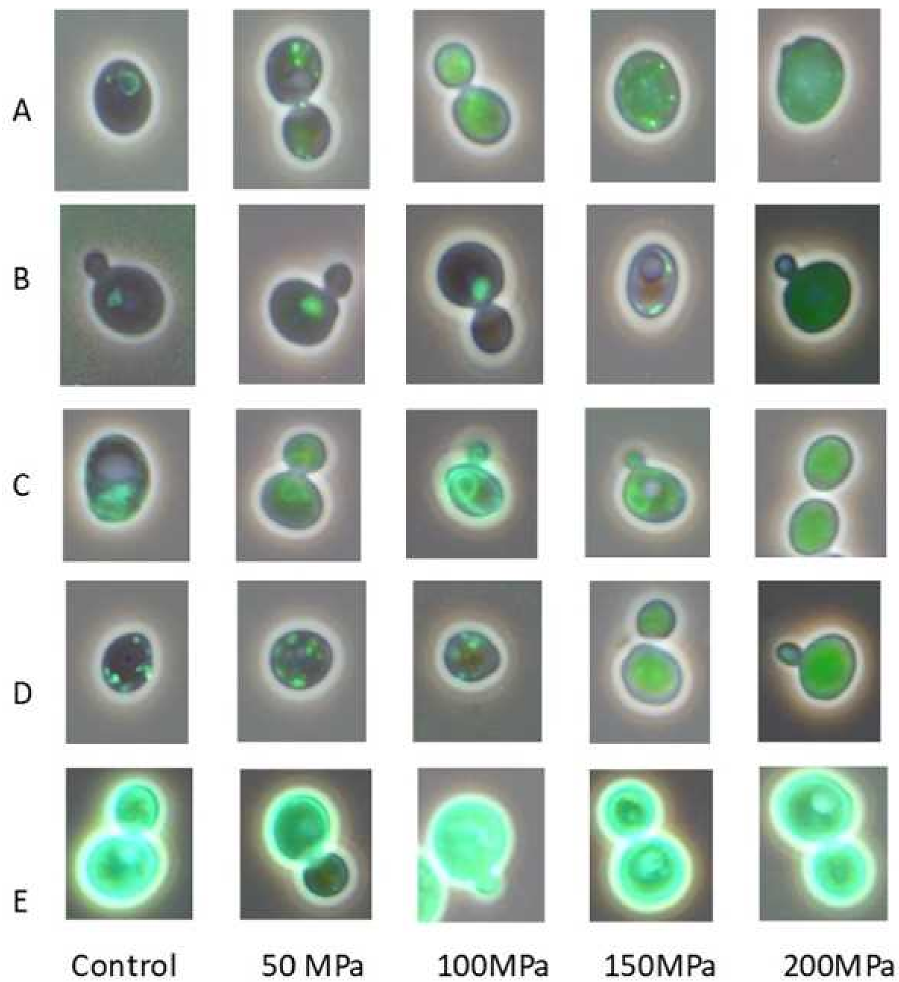

Fig.5 Visualized structures of organelles after pressure treatment. The images were visualized by fusing microscopic image and fluorescent microscopic image. A; endoplasmic reticulum, B; Golgi body, C; nucleolus, D; nucleus membrane, E; cellular membrane

Typical fluorescent microscopic appearance of nucleolus membrane labeled yeast strain (BY4741 GFP-Nic96 YFR002W) after various pressure conditions were shown in Fig. 5A. Those structures seem to ripe after 50MPa and to be teared after 100MPa. This strain showed approximately 50% CFU after pressure treatment and less than 20％ CFU after 100MPa pressure treatment (Fig.3). Those images are well agreed with CFU after the corresponding pressure treatment. Fig.5B shows protein localized in nucleolus (BY4741 GFP-Rox3 YBL093C) and it seems no significant difference up to 150MPa. These images do not agree with CFU after the pressure. In contrast to nuclear membrane, the fluorescent microscopic images show the stability up to 150MPa. The Rox3 gene encodes a protein playing the RNA polymerase II mediator complex. The RNA polymerase II mediator complex has huge structures and can be constitute of nucleolus in nucleus. Nucleolus structure is not constituted by membrane structures. Thus, the Rox3 mediated structure can be observed stably up to 150MPa.

Typical fluorescent microscopic appearance of endoplasmic reticulum labeled strain (BY4741 GFP-Pho86 YJL117W), Golgi body labeled strain (BY4741 GFP-Sec7 YDR170C), and cellular membrane labeled strain (BY4741 pPTR2-4K＞R-GFP) were shown in Fig. 5C, 5D, and 5E. It seems that endoplasmic reticulum and Golgi body keeps structures up to 100MPa and do not correlated with CFU (Fig. 3). Cellular membrane kept its structure up to 200MPa and this structure could not be the critical.

3.3 Leakage of intracellular substances from the cells by high pressure

UV-absorbing substances at 260 or 280 nm began to release the substance from the industrial yeast strain O-39 even at low pressure of 100 MPa. With rising pressure up to 200 MPa., the leakage substance were gradually increased, and treated cells at 200 MPa, it was same as treated cells with heat at 100°C (data not shown). UV-absorbing substance was almost 10 times when the cells treated hydrostatic pressure 500 MPa compared at treated cells 200MPa (Fig. 6).

Viability of the cells treated up to 200MPa were drastically decreased and treated 300MPa, viable cells were almost zero. This results suggested that cell treated by hydrostatic pressure destroyed especially at membrane, and leaked the intracellular substances from the cells and dead.

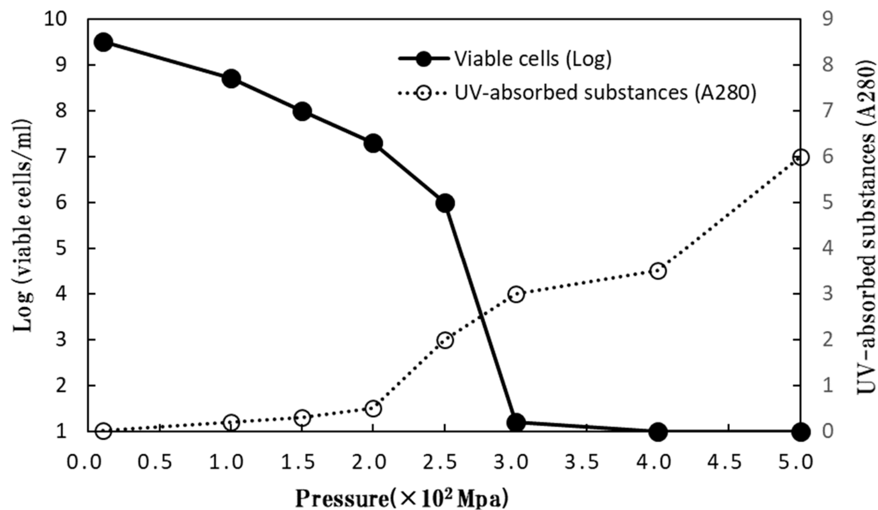

Fig. 6 Leaked ultraviolet (UV)-absorbing substances and cell viability of *S. cerevisiae* cells exposed to various pressures for a constant duration of 10 min at 25°C (S. Shimada et al. 1993).

Leakage another substances such as glutathione and metal ion were increased as up to pressure. Low-molecular-mass compounds including peptides in the cells, glutathione (GSH), which was a biologically active peptide, also began to be released at increasing pressure (Table 4).

Table 4. Leaking of glutathione (GSH) from cells of *S. cerevisiae O-39* after pressure at 25°C or -20°C (S. Shimada et al. 1993)

Pressure conditions	Release of GSH (nmol/ml)
0.1 Mpa, boiling, 100℃, 5 min	520
300 Mpa, 25℃, 10 min	28
400 Mpa, 25℃, 10 min	546
500 Mpa, 25℃, 10 min	463
600 Mpa, 25℃, 10 min	427
200 Mpa, -20℃, 3h[a]	931

[a] After pressure at room temperature, samples were cooled and maintained at -20℃ under pressure conditions, and then exposed to room temperature for 30 min, and decompressed

When cells were treated with pressure above 400 MPa, the amount of GSH released from the cells was almost same as treated 100°C for 5 min. Moreover cells treated 200MPa -20°C for 3h, the amount of GSH released was approximate twofold.

Examining the release of metal ion from inside the yeast cells by high pressure, compared with heat treatment, the amount of minerals treated 300MPa was almost 4 times, and 600MPa 6 times (data not shown). Therefore high pressure treatment may be very useful for the removal or separation of internal substances such as metal ion, amino acid and peptides without breaking the cells.

3.4 Effect of high pressure on viability of yeast cell *S. cerevisiae* and detection of variant cells on dye plate

Dye plate contains two kind of different dye pigment in nutrient medium and the use of the pigment varies according to the kind of the yeast strain, so it is easily distinguished with the color of the colonie which appeared onto dye plate. We confirmed whether strains were affected some kind of hereditary influences by high pressure, we examined cells in latter of exponential growth phase were treated at high pressure, and applied on dye plate which could be identified variant by colony color.

Parent diploid strain *P-544* on dye plate were violet but cells treated at 200MPa for 10 min. and applied onto dye plate, several colors of colonies and small colonies were appeared. (Fig.7) (K. Hamada 1992). A similar phenomenon was also observed in haploid strains (Fig. 8).

Fig.7 Evidence of induction of variants (*arrow*) by high pressure treatment. exponential growth phase culture of *S. cerevisiae P-544* were treated without (a) and with (a) pressure 200MPa. for 10min at 25°C. Arrows are variant colonies.

Fig.8 Evidence of induction of variants (*arrow*) by high pressure in the haploid cells *HF399s1*.

No pressure (above); with high pressure at 200MPa. (below)

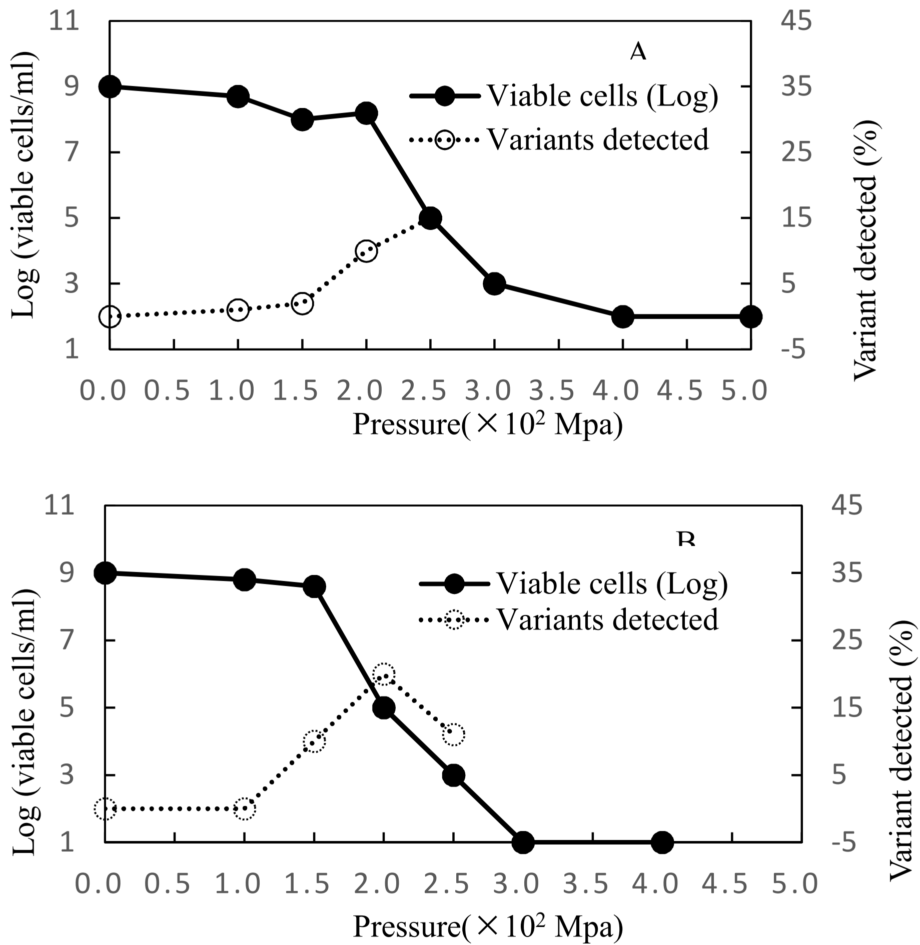

Fig.9 Effect of the magnitude of high pressure on cell survival and the appearance of variants detected from the diploid *P-544* (A) up, haploid *HF399s1*(B) below.

We examined the effect of the magnitude of high pressure on cell survival and the induction of variants from the diploid cell *P-544* and haploid cell *HF399s1*. As shown in Fig.9 A, above 150 MPa, diploid strain *P-544* displayed a gradual decrease in cell viability, while it was drastically inactivated at pressure over 400 MPa.

On the other hand, haploid strain *HF399s1* showed a slightly different survival curve compared with diploid strain (Fig.9B). Above 100 MPa, a slightly decrease in cell viability was observed, but up to 250MPa, this strain was fully inactivated. In addition, pressure treatment over 100 MPa, variant colonies from *P-544* and *HF399s1* appeared and increased in frequency up to 250 MPa. The maximum occurrence of variants in appearance onto dye plate was 14.5% for *P-544* and 18.3% for *HF399s1* respectively. The variant occurrence rate of both strains in exponential growth phase was higher than stationary phase (data not shown).

Table 5 Properties of the variant *P-544V1* derived from *S. cerevisiae P-544* after pressure treatment at 250 MPa for 10 min at 25°C.

Strain	*MAT*	Colony color on dye plate	Cell size[a] (μm)	DNA content[b] (μg/10^9 cells)
P-544	a/α	Violet	4.18	27.5
P-544V1	a/a/α/α[c]	Red/blue	5.51	63.6
P-559	a/a/α/α	Violet	5.69	55.9

[a] After pressure treatment, culture samples were taken, diluted, and spread on a dye plate, and then incubated at 25℃ for 4-5 days. The variant *P-544V1* colony formed on a dye plate was picked out and its cell size calculated by using the Elzone particle counter 80XY

[b] The variant P-544V1 colony formed on a dye plate was picked out and then cultured on YPD medium at 30℃. After incubation for 15h, cells were harvested and their DNA contents measured by the methods of Aigle et al. (1983)

[c] Supposed mating type

Table 6 Properties of the variant *HF 399s1V4* and its diploid or triploid in crosses

Strain	Combination	MAT	Colony color on dye plate	Cell size[a]	DNA contents (μg/10^9 cells)
HF399s1	Original haploid	α	Violet	3.79	n.d[b]
HF399s1V$_4$[c]	Variant	α/α	Red/blue	5.45	27.8
PA-602	*HF399s1×Ha112(a)*	a/α	Pale blue	5.93	33
PA-603	*HF399s1V*$_4$*×Ha112(a)*	a/α/α	Pale blue	6.15	44

[a] Measured directly on a light microscope/Image Analyzer

[b] Not determined

[c] The *HF399s1V*$_4$ variant was isolated from colonies with a red/blue color on a dye plate

[d] Supposed mating type

Table 5 shows the properties of variants from strains *P-544*. The one variant *P-544V1* isolated from *P-544* had an average diameter around 1.3 times larger than the parent cells. In addition, chemical analysis revealed that *P-544V1* contained 63.3μg of DNA per 10^9 cells, which was almost double the value of 27.5μg of DNA per 10^9 cells of parental cells. Cell diameter and DNA content of *P544V1* was the same value compared with *P-559* strain known to be tetraploids. On the other hands, all variants colonies from diploid strain *P-544* inherited their original mating type (a/α) of the cell (*P-544V1*). The chromosomal karyotype was examined with pulsed field electrophoresis after acquiring cells of different colors in the dye plate, and the appearance of cells with different karyotype was observed (data not shown). Similarly, the variant *HF399s1V4* isolated from the haploid *HF399s1* by pressure treatment were average diameter around 1.4 times that of parental strains. Cellular DNA contents of their variant cells were almost double compared with their parent cell (Table 6).

Variant strain *HF399s1V4* and *HF399s1* were easy to conjugate with *Ha112 (MAT a)*, which was known mating type. As a result, mating type of *HF399s1V4* derived from *HF399s1* was same as parental strain (*MAT α*).

Table 7 Occurrence of variant cells originated from diploid and haploid strains of *S. cerevisiae* after pressure treatment at 200 MPa or 250MPa

Strain	Before pressure treatment		After pressure treatment		
	Color on dye plate	Ploidy	Color on dye plate	No. of variants/ no. examined	No. of large sized/ no. variants
P-540	Violet	2n		24/500	23/24
			Violet	476	
			Red/blue	24	
P-544	Violet	2n		14/94	14/14
			Violet	80	
			Red/blue	14	
O-39	Violet	2n		15/700	14/15
			Violet	685	
			Red/blue	15	
HF391s1	Violet	n		11/60	11/11
			Violet	49	
			Red/blue	11	
HF399s18	Violet	n		10/198	9/10
			Violet	88	
			Red/blue	10	

In addition to *P-544* , *HF399s1* (α haploid), other strains, such as *P-540* (a/ α diploid), *O-39* (a/α diploid) and *HF399s18* (a haploid) were treated with pressure at 250MPa or 200MPa for 10 min and plated on a dye plate and incubated. As a result, variant colonies, which were stained red/blue were almost all of large-sized cells under a microscope. (Table 7). All variant colonies from haploid strains inherited their original mating type. On the basis of cellular DNA content, it was determined that variant colonies which stained red/blue after pressure treatment had almost double the DNA content of their parent cultures (data not shown).

We investigated appearance of similar variant colonies with large-sized cells derived from other strains by hig pressure.

3-5 Effect of magnitude of pressure on viability of yeast cell *S. pombe* and detection of variant cells on dye plate compared with *S. cerevisiae*

We first investigated whether a dye plate-colony color assay system for detecting variant in the pressure treated cells of *S. pombe* would be useful, as described in *S. cerevisiae*, and this phenomenon applied to fission yeast *S. pombe* laboratory strains which was closed to mammalian cells (K. Hamada 1992). This plate method was also provided a convenient colony color assay at the fission yeast *S. pombe*.

Stationary growth phase cultures of *S. pombe JY1* (*L972h*$^{-}$) treated by high pressure at 200 MPa., and this culture was spread onto a dye plate, several colors of colonies were appeared (data not shown). This pink-violet colonies from JY1 (or JY3, JY333 or JY 334) cells treated by high pressure, appeared on the dye plate were clearer than that of the same cells appeared on the phloxin B plate.

These variant colonies from the strain were very stable, and gave rise to large colonies unchanged in the color when subsequently transferred to fresh dye plate (data not shown).

Table 8 Effect of magnitude of high pressure on the induction of variants.

Pressure stress	No. of variants detected (V)			Total no. of clonies examined (T)			V/T (%)		
	JY1	*JY3*	*JY334*	*JY1*	*JY3*	*JY334*	*JY1*	*JY3*	*JY334*
100 Mpa, 25℃, 10 min	2	2	1	100	150	62	2.0	1.3	1.6
150 Mpa, 25℃, 10 min	6	12	15	80	213	180	7.5	5.6	8.3
200 Mpa, 25℃, 10 min	103	109	23	266	308	62	38.7	35.4	37.1

We also examined the effect of the magnitude of pressure stress on the formation of variants. As shown in Table 8, the degree of variabilities in the appearance of variants varied in pressure from 100 to 200 MPa. After pressure stress over 100 MPa, variant from these colonies

began to appear and increased in frequency up to 200 MPa. The maximum percent of cell population of variant colonies from *JY1*, *JY3* and *JY334* after pressure stress at 200 MPa were 38.7, 35.5, and 37.1, respectively. These values were almost double that obtained for the variant colonies from the pressure-stressed haploid cells of *S. cerevisiae* under the same condition (K. Hamada 1992).

We examined the effect of magnitude of high pressure on cell survival of the parental strain *JY1* and variant *JY1-V1* which parent strain was treated by high pressure and picked up from a dye plate.

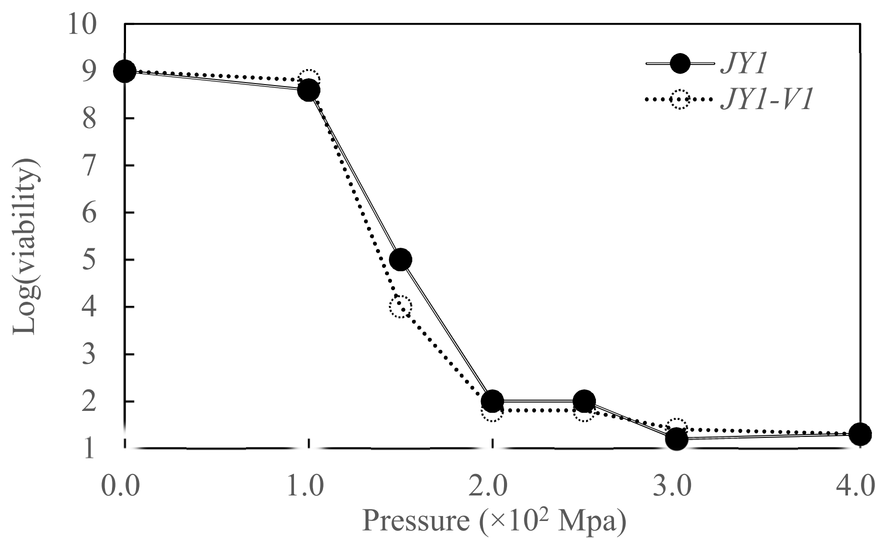

Fig. 10 Comparative cell survival of the parent *JY1* and the variant *JY1-V1* with large-sized cells culture when subjected to various magnitude of applied pressure.

Fig. 10 showed the effect of the magnitude of high pressure on cell survival of two strains, *JY1* and *JY1-V1* which was picked up from dark-violet colonies formed on a dye plate after pressure stress at 200 MPa. As shown in Fig. 10, both strains showed a similar survival curve, and above 100 MPa, they displayed a drastic decrease in cell viability. This result

indicated that *S. pombe* cells were generally more sensitive to pressure stress than *S. cerevisiae*.

Table 9 Properties of the variant *JY1-V1* derived from *S. pombeJY1* (*L972h*$^-$, wild type) after pressure stress at 200 MPa for 10 min at 25°C

Strain	Genotype	Cell sizea (µm)	DNA content (µg/10^9 cells)	Colony color
JY1	h^- (L*972*, wild type)	9.8±2.2	21.3	Pink
JY334	h^+ *ade6-M216 leu1*	nt[b]	23.6	Pink
JY1-V$_1$	h^-/h^- (variant)[c]	15.0±3.4	44.8	Violet
JY276	h^-/h^- *his2/his2 ade6-M216/ade6-M210*	14.2±3.2	50.3	Violet

[a] Average cell length: mesured directly on alight microscope/image analyzer.

[b] Not tested.

[c] Assumed type

From the 103 variant colonies formed on a dye plate after pressure 200 MPa, the *JY-1V1* colony was randomly chosen to determine ploidy. Ploidy was evaluated by cell size (cell length) and DNA content. Table 9 showed the properties of variant *JY1-V1* from the haploid strain *JY1*. The single variant *JY1-V1* cell isolated from *JY1* more closely resembled both in cell length and DNA content of the parental diploid strain *JY276*. To investigate the variant *JY1-V1* cell could be defined as a homozygous diploid, this variant and the standard haploid strain *JY334* of mating type h^+ were crossed pairwise with each other.

As expected, they were able to cross with each other. Similarly, other variants *JY1-V2* and *JY1-V3* with large-sized cells (not shown), which were isolated from *JY1* under the same condition, were able to cross with opposite mating type of *JY334*.

3.6 Properties of variant cells induced by high pressure of *S. cerevisiae* and *S. pombe*

Variants colonies from *JY1*, *JY3* and *JY334* were almost all considered of large sized cells (Table 9). These variant colonies were very stable, and gave rise to large colonies did not change in color when continue transferred to new dye plate. We also examined the effect of the magnitude of pressure stress on the formation of variant cells. The degree of variability in the appearance of variants varied in pressure from 100 to 200MPa.

After pressure stress over 100MPa, variant colonies appeared and increased in frequency up to 200MPa. The maximum percent of cell population of variant colonies from them were up to 30% (Table 8). These values were almost double compared with haploid strain of *S. cerevisiae* under the same condition. The magnitude of pressure stress on cell survival of *JY1* (haploid) and *JY1-V1* (derived from *JY1*) showed a similar survival curve regardless of difference in cell size.

3.6 Genetic analysis of variant yeast cells by high pressure

We investigated the ploidy of variant cells, we performed random spore isolation, and mass - mattings. As shown in Table 10, two variant colonies *P-540V1* and *P-544V1* were grown, sporulated, and their random spores scored for segregating markers of mating types.

In the variant *P-540V1* from *P540*, mating types showed 77 non: 11 a: 12α: pattern of segregation in 100 spore clones tested, another variant *P-544V1* from *P-544* showed 66 non: 16 a: 18α: pattern of segregation. The observed segregation ratios of mating types found both the above variants were not significantly different from the theoretical ratio, which is 4 non: 1 a: 1α: as found with an a/a/α/α genotype (χ^2=4.89, *2df*, P>0.05, for *P-540V1*: χ^2=0.14, P>0.90, for *P-544V1*).

Table 10 Segregation of mating types in variants *P-540V1* and *P-544V1* induced by pressure at 250 MPa for 10 min at 25°C

Strain	Segregants	Segregation of mating type a : α : non	Estimated gene constitution based on χ^2 analysis
P-540, original	48	23 : 25 : 0	a/α
P-540V1	100	11 : 12 : 77	a/a/α/α
			χ^2=4.89 *2df*, p>0.05
P-544, original	44	23 : 21 : 0	a/α
P-544V1	100	16 : 18 : 66	a/a/α/α
			χ^2=0.14 *2df*, p>0.90

Since all non-meter from the same variant *P-540V1* and *P-544V1* cells could sporulate well, they were identified as a/α diploid. The variant *HF399s1V4*, derived from haploid *HF399s1* (α), was crossed to a haploid *Ha112* (a) and the resulting hybrids were selected, and tested random spore isolations (Table 11).

Table 11 Random spore analysis with hybrids formed in crosses between *HF399s1*, *HF399s1V4* and the haploid tester strain *Ha112*

Strain	Combination	Segregants	Segregation of mating types a : α : non	Estimated gene constitution based on χ^2 analysis
PA-602	*HF399s1* × *Ha112*(a)	81	39 : 42 : 0	a/α
PA-603	*HF399s1V4* × *Ha112*(a)	95	16 : 48 : 31	a/α/α
				(χ^2=0.023, *2df*, P>0.975

In the strain *PA-603*, mating types showed 31 non: 16 a: 48α segregations in the 95 spore tested. These segregation data for *PA-603*, which fitted a theoretical 2 non: 1 a: 3α ratio, indicated that this strain was triploid with a/α/α genotype (χ2=0.023, *2df*, P>0.975). Consequently, all the data (including the segregation patterns of the mating type, cell size and the DNA content) used for the identification of ploidy clearly indicated that *P-540V1* and *P-544V1* were tetraploids with a/a/α/α genotype (Table 10), while *HF399s1V4* was a homozygous α/α diploid (Table 11).

To identify ploidy in the variant cells from *S. pombe*, by hydrostatic pressure, we carried out random spore analysis. The variants *JY1-V1* and *JY1-V2* were mated with the opposite mating type of strain *JY334*, sporulated on MEL plates, and subjected to this analysis (Table 12).

Table 12 Random spore analysis with hybrids formed in crosses between *S. pombe JY1, JY1-V1* and the haploid tester strain *JY334 (h^+)*

Strain	Cross	segregants	segregation of mating type h^- : h^+ : h^-/h^+	Estimated gene constitution based on χ^2 analysis
HPI-01	*JY1(h^-)* × *JY334 (h^+)*	40	23 : 17 : 0	h^-/h^-
HPI-11	*JY1-V_1(h^-/h^-)* × *JY334(h^+)*	46	25 : 7 : 14	$h^-/h^-/h^+$
				(χ^2=1.00, *2df*, P>0.50

In the hybrid *HPI-11* formed in crossed with *JY1-V1* and *JY334*, mating types showed 14 h^-

/h$^+$: 7 h$^+$: 25 h$^-$ sergeants' in 46 spore clones tested. From the results of the χ^2-test, the observed segregation ratio of mating types formed in the above hybrid, *HPI-11* was not significantly different from the theoretical ratio, which showed *3 h$^-$/h$^+$:1 h$^+$: 2 h$^-$* segregation with genotype *h$^-$ / h$^-$ /h$^+$*. This also suggested that the variant *JY1-V1* was a homozygous diploid with an *h$^-$/h$^-$* genotype. From the results of the χ^2-test, the observed segregation ration of mating types. *HPI-01* was the diploid and *HPI-11* was triploid cell according of the segregation patterns of mating type.

Table 13 Random spore analysis of segregations of the marker *ade6* allele and triploid crosses

Strain	Cross	Total no. of segregants	Genotype	Observed phenotypic segregations
				Ade$^+$: *Ade$^-$*
HPI-01	*JY1(h$^-$)* ×*JY334* (*ade6 leu1 h$^+$*)	59	*ade6*	34:25 (2:2)[a]
HPI-11	*JY1-V$_1$*(*h$^-$/h$^-$*)[b]×*JY334* (*ade6 leu1 h$^+$*)	63	*ade6*	53:10 (5:1)[c]
HPI-12	*JY1-V$_2$*(*h$^-$/h$^-$*)[b]×*JY334* (*ade6 leu2 h$^+$*)	98	*ade6*	81:17 (5:1)[d]

Figures in parentheses are theoretical value expected from a diploid or a triploid which has the auxotrophic markers

[a] χ2=0.028, 1 df, P>0.80.

[b] Supposed mating type.

[c] χ^2= 1.612, *1df,* P>0.20.

[d] χ^2= 0.036, *1df,* P>0.80

The segregation patterns of the auxotrophic marker such as *ade6* or *leu1* in these hybrids were also analyzed. Hybrids *HPI-11* and *HPI-12* were sporulated on MEL plates and segregated on YPDS plates to form colonies. Replica plating on these colonies on SD (plus adenine and leucine) plates, allowed examinations of the segregation of additional auxotrophic markers. In one case, for three crosses, *Ade$^-$* sergeants' were subjected to random spore analysis after sporulation for 3 days on MEL plates.

As shown in Table 13, the ratio of *Ade$^+$* to *Ade$^-$* for *HPI-11* and *HPI-12* were 53:10 and 81:17, respectively, both consistent with the expected ratio of an allele of *ade6* gene (5 *Ade$^+$*:1 *Ade$^-$*) in a triploid zygote.

Thus, like *S. cerevisiae*, in the fission yeast *S. pombe* direct diploidization appears to have been established using the procedures described by Broek et al. (Broek, D 1991), Moreno et al. (Moreno, S 1994), and Yoshida et al. (Yoshida, M 1990). The appearance of multiple strains and mutants by hydrostatic pressure stress was a completely new phenomenon and was one of new mutation methods. Pressure treatment in combination with dye plate was easily carried out and more effective for producing high yield of tetraploids or homozygous diploids. In general, this method suggests that high pressure is potential inducing agent of polyploidy in culture of *S. cerevisiae* and *S. pombe*.

3.8 Why did polyploidy variants appear by high pressure

Yeast cells under high pressure of 200MPa or more appeared with a high frequency of polyploid with damage of other intracellular organelles. Osumi et al. investigated the mechanism for induction of polyploidy by high pressure. The microtubules of *S. cerevisiae* and *S. pombe* were studied by conventional and immunoelectron microscopy (M. Osumi 1996).

Even at 100MPa, nuclear membrane was disrupted, as pressure increasing mitochondria and cytoplasmic substances changed dramatically and cellular organelles could hardly be detected. Immuno-electron microscopy confirmed that the microtubules were damaged by high pressure, and confirmed *S. pombe* cells were more sensitive than *S. cerevisiae*. The damage to spindle pole bodies, microtubules and the nuclear membrane were followed by breakdown of nuclear division apparatus and inhibition of nuclear division.

They investigated the cell cycle-specific organization of microfilaments and microtubules in *S. cerevisiae*, *S. pombe* and *C. tropicalis* were altered by exposure to high pressure of 50-150MPa, and their complete disassembly was observed at 150-300MPa. (H. Kobori 1995).

Similar morphological changes in the cytoskeleton were caused in these yeasts by acceleration of pressure stress, but their sensitivity were differed. *C. tropicalis* and *S. pombe* were more sensitive to pressure stress. These results suggested when cell division was initiated, cell cycle-specific organization of microfilaments and microtubules were damaged, and occurrence of polyploids of *S. cerevisiae* and *S. pombe*.

The appearance of polyploidy suggested that the fission mechanism was affected by high pressure. Comparing *S. cerevisiae* with stationary phase and with exponential phase, it has also been found that the polyploid appearance rate was significantly higher in the exponential phase (data not shown). In order to clarify this phenomenon, immunoelectron microscopy (immunostaining using anti-tubulin/ monoclonal antibody rat IgG as a primary antibody, goat anti-rat IgG labeled with gold particles (10nm) as a secondary antibody) was performed and

observed.

Microtubules and spindle pole body were observed in the nucleus in normal cells not given high pressure stress, and the localization of tubulin was observed along the microtubules. Localization of tubulin was observed even at 100MPa., microtubules were also observed, but spindle pole body was not observed. At 150MPa., gold particles indicating tubulin localization were observed but the microtubules disappeared. At 200MPa., aggregation of chromatin in the nucleus was observed, cracks and voids were observed, and at 300MPa. the nuclear membrane was markedly damaged.

The above results indicate that the change in localization of microtubules, spindle pole body and actin fibers disappeared and disintegrated and tubulin inhibited the normal progression of cell cycle, along with the collapse of the nuclear membrane, causing the formation of polyploids. Similar cases are also introduced in the test using *C. tropicalis.*

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3.9 Efficient methods for separating ascospores from sporulating cultures in *S. cerevisiae* by high pressure and improving new method for industrial baker's yeast strain

Nakatomi et al. have proposed an efficient method for separating ascospores from the mixture of vegetative cells and ascospores using the difference of tolerance due to the high pressure of the vegetative cells and ascospores (Y. Nakatomi 1993).

Table 14 Loss of viability of various *S. cerevisiae* strains after exposure of vegetative and sporulated cultures to high pressure of 300MPa. for 10min at 25°C.

Strain	Growth conditions	Cell concentration		Sporulation rate(%)
		Before pressure	After pressure	
HF69 (a/α)	Vegetative	4.3×10^{7}	<10	
	Sporulation	1.2×10^{7}	1.6×10^{5}	25.2
FRI413	Vegetative	4.5×10^{7}	<10	
	Sporulation	9.6×10^{6}	9.3×10^{3}	1.1
FEF11 (a/a/α/α)	Vegetative	2.6×10^{7}	<10	
	Sporulation	3.9×10^{6}	3.2×10^{5}	21.4
HF399 (a/α)	Vegetative	3.6×10^{7}	1.4×10^{3}	
	Sporulation	1.1×10^{7}	8.2×10^{5}	45.7
O-39	Vegetative	3.7×10^{7}	<10	
	Sporulation	1.3×10^{7}	<10	<0.01

[a] Vegetative culuture were prepared by growth on YPD medium at 30℃ for 48 h. Sporulation culture were prepared on a solid sporulation medium at 25℃ for 5 days. Prior to pressure treatment all cultures were resuspended in distilled water.

[b] Cell concentration was estimated as the colony forming unit/ml on a dye plate after incubation at 25℃ for 5days. All colonies, which stained red/blue, dark blue, pale blue, or violet, formed on dye plates after pressure treatment or before treatment were counted.

Table 14 showed ascospores were much more resistant to high pressure than non-sporulated cells and vegetative cells. Moreover, an analysis of meiotic segregation of mating types was carried out in random spore samples from several pressure-treated ascospores at 300MPa. for 10min by the method of Spencer (J. F. T. Spencer 1988). Segregation of mating types were not

different from that obtained by conventional techniques. For *S. cerevisiae* genetics, the most useful aspect of high pressure treatment was that it was rapid, convenient and could be used readily to select mono-spore cultures on dye plates. Nakatomi et al. suggested this new procedure was simple, time saving and more efficient one step method for random spore analysis in the industrial yeast.

In general industrial baker's yeast strains are useful to produce good feature of bread. They should have several useful properties such as strong gassing power at high sugar dough, frozen dough resistance and producing rich flavor. Moreover, they should have manufacturability, such as high yield, high productivity, high temperature resistance and fast growing in the fermentation tank.

It is very common to developed a new kind of yeast strains it has been basically indispensable for using parental strains by rare-mating or sexual mating method. But using hydrostatic pressure method, we are able to easily and efficiently improve to make polyploid cells from diploid cells and segregated and screening. For developing of industrial baker's strains it is very important issues to retain useful properties and eliminate bad property (growth rate and dehydration efficiency is low, and not resistant high temperature around 37°C) can be possible.

If we can get excellent industrial strains having several useful properties but it does not good manufacturability, first we make tetraploid cells by hydrostatic pressure and sporulation of this strain and screening that retain useful properties and eliminate bad properties such as low efficiency etc. In fact author could be successful to develop the new baker's yeast in a short period of time by using hydrostatic pressure method and it has been used in the bakery market.

4. DISCUSSION

Yeast cells were damaged by high pressure up to 200MPa., especially the structural impact on the membrane system, particularly the nuclear membrane even at 100 MPa (Fig. 2b). Pressure increasing above 400 MPa., cells were strikingly affected in their inner structure and leakage several inner substance such as mineral, amino acid and glutathione. This results indicate high pressure treatment may be very useful for removal or separation of internal substances such as metal ions, amino acid pools and peptides from yeast cells without breaking the cells.

S. cerevisiae were effected by high pressure, viability of the cells were gradually decreased with increase of the pressure, and up to 300MPa., all of cells were inactivated. The results of *S. pombe* suggested similar phenomenon observed but *S. pombe* was more effected rather than *S. cerevisiae* by high pressure (M. Sato 1996). Effect of high pressure on yeast cells induced high frequency of variant cells on a dye plate. These variant cells were easy to distinguish as parent cells on a dye plate, and become bigger than parent cells. Random spore analysis of these variants showed they were homozygous diploid from haploid strain and homozygous tetraploid from diploid strains.

The analysis of chromosomal patterns of these variants, a little number of variant defected chromosomal changes in plus field electrophoresis, so high pressure can be used for physical mutagenesis. This phenomenon was appeared in fission yeast *S. pombe*. As for the mechanism of polyploidization by pressure stress, we suggested previously (K. Hamada 1992), that in the budding yeast *S. cerevisiae*, cytoskeletal elements including microtubules and microfilaments, which were strongly related to nuclear division apparatus or cell polarity in the cells, were completely disrupted which resulted in the promotion of polyploidization.

In the yeast *S. cerevisiae* and *S. pombe*, direct induction of polyploidization has been obtained in a variety of ways including rare-mating methods (Gunge N 1972), protoplast fusion (Emis VCC 1960), light irradiation (Landau JV 1970), treatment with MBC. Many hours are

necessary to test these treatments in yeast, especially for the induction and selection of polyploid clones from diploids. But diploid cells induced by these procedures described above are generally unstable. Our results, however, demonstrated that not only the difficulties in diploidization but also the stability of these cells combination with a dye plate, and induced by hydrostatic pressure may be a similar phenomenon to the duplication of the chromosome set observed in fishes.

Pressure-treatment and combination with a dye plate method are easily carried out and more effective for producing high frequency of tetraploid or homozygous diploid clones. In general, this method suggests that high pressure is a potent inducing agent of polyploidy in cultures of *S. cerevisiae*.

H. Kobori et al. studied that cells were treated with high pressure at 100 MPa. on yeast cells, spindle pole bodies disappeared, at 150 MPa, the deposition of gold particles for anti α-tubulin was noticed in the nucleus, although the filamentous structure of microtubules was lost (H. Kobori 1995).

These results showed that elements of nucleus division apparatus were susceptible to pressure stress, particularly spindle pole bodies and microtubules. The damage of spindle pole bodies, microtubules, and nuclear membrane caused by pressure stress was followed by the inhibition of nuclear division. After release of pressure, the spindle pole bodies and microtubules of pressurized cells at below 200MPa regained their normal appearance at 24h. Fluorescence microscope studied on microtubules and microfilaments revealed that the cytoskeleton was one of the most susceptible elements to hydrostatic pressure in the subcellular structure of *S. cerevisiae*.

Formation of polyploidy cells by hydrostatic pressure has been documented in many higher eukaryotes (Dasgupta. S 1995), (Sato. M 1995) . Onozato confirmed that the breakdown of the mitotic spindle after application of hydrostatic pressure was caused by formation of polyploidy in Salmonid eggs. The mitotic spindle of *S. cerevisiae* partially or completely disappeared at the same range of pressure (200-250 MPa.) at which formation of polyploidy was observed at high

frequency (Hamada, K. 1992). Thus, the same phenomenon observed in higher eukaryotes probably facilitated the induction of polyploidy in *S. cerevisiae*. Recoverability of the damaged mitotic spindles and nuclear membranes might also contribute to the frequent formation of polyploidy in this yeast.

M. Sato, et al. (1996) investigated that effect of high pressure on ultrastructure, microtubules and microfilaments of *S. pombe* using fluorescence microscopy, conventional electron microscopy and immunoelectron microscopy. Cells were treated at above 100 MPa, the nuclear membrane was disrupted, and α-tubulin was lost in most of the cells at same pressure treatment. The gold particles for anti α-tubulin were not visible in the cells at the same level. Cell cycle specific actin distribution was lost even at 50 MPa., thick actin cables appeared at 100 MPa.

These results suggested that *S. pombe* cells were more sensitive than S. cerevisiae cells. They suggested the damage to microtubules and nuclear membrane caused by high pressure was thought to be followed by break down of nuclear division apparatus and inhibition of nuclear division. This damage might contribute to the frequent formation of polyploidy in *S. pombe*.

They reported previously the induction of polyploidy in *S. cerevisiae* is accompanied by the breakdown and reassembly of spindle microtubules (Osumi, M. 1992). The induction of polyploid cells by pressure stress therefore, apparently occurs widely in yeasts and lower eukaryotes.

We think that the possibility of duplication of chromosome sets in yeasts were easy to get by high pressure. This method is easy to use for developing and improving the industrial yeast strains, such as baker's yeast and brewing yeast strains. This technique is easily applied and strikingly effective for developing industrial yeasts producing a high yield of homozygous diploids in the budding yeast *S. cerevisiae* and fission yeast *S. pombe*.

Our research indicates that it is very easy and efficient to make the homozygous diploid and tetraploid cells by hydrostatic pressure on industrial yeast strains and apply to improve an excellent yeast strains combined high pressure and a dye plate method.

5. CONCLUSION

Yeast cells of *S. cerevisiae* were affected by increasing pressure, and internal substances such as metal ions, amino acids and peptides leaked from the cell. Observing the organs of the cells affected by high pressure using electron microscope showed that cell membrane and nuclear membrane were affected even under a relatively low pressure at 100 MPa. and cells which destroyed parts of the structures of nuclear membrane was around 14% of total cells observed. Similar results were obtained for cells stained *Candida tropicalis* with 4, 6-diamidino-2-phenylindole (DAPI).

In order to observe the influence of high pressure on internal structure of the cells, intracellular organ such as *S. cerevisiae* BY 4741 of Golgi bodies and nuclear membranes etc. were stained with GFP were prepared and the colony forming units and structural changes of each organelles were observed with a microscope. As a result, it was found that the nuclear membrane was damaged under fragmentary at 50 MPa, and cells were inactivated. The above results showed that the membrane of the cells, particularly nuclear membrane, was susceptible to high pressure, and related to inactivate.

Studies using budding yeast and fission yeast, cells surviving due to high pressure, those in which a part of chromosomes were missing or those with two sets of chromosomes were highly appeared. When cell suspension subjected to pressure treatment was applied to nutrient medium added dye (dye plate) and incubated at room temperature, many colonies and small colonies (presumed to be respiratory deficient strains) apparently different in color tone from the parent strain appeared onto the plate. In order to investigate the relationship between pressure and appearance rate of mutant strains, when high pressure was applied to diploid strain *S. cerevisiae P 544* (*MAT a / α*), the appearance rate of colonies with different color tone were increased according to increase magnitude of pressure treatment. Pressure treatment at 200 MPa., the appearance rate of variant was around 20%. The cell diameter of *P 544* V_1 which was one of the

obtained variant was 5.51μm, which figure was close to 5.69μm of tetraploid strain of P *599* strain and the diameter of diploid parent strain *P 544* was 4.18μm. The DNA content of $P544V_1$ was 63.3μg per 10^9 cell, this figure was almost double of DNA content of parent strain *P544* (27.5μg per 10^9 cells).

Chromosomes were extracted from the cells obtained by mutants containing $P544V_1$, one of the variants of the chromosome migration pattern was confirmed same as parent strain by electrophoresis and another variant was confirmed that the chromosomal migration patterns was different.

The separation ratio of MAT by random spore separation analysis of P544V1 was *a: α:* non = 1: 1: 4, which was the same as the separation ratio of tetraploid. The variant strains obtained from the above results were found to be self-tetraploid strains of *MAT (a / a / α / α)*. The same phenomenon was observed also when treating the haploid strain $HF399s_1$ (*MATα*) at 150 MPa. pressure treatment, all of the variants that appeared onto the dye plate were *MAT (α / α)* and inherited the original mating type. It was suggested that the appearance rate of the mutants were related to the cell cycle, since the appearance rate of the s became higher when using cells in logarithmic growth phase. These results indicated that when cells undergoing cell division were subjected to pressure, tissues related to nuclear division such as tubulin and spindle pole body were affected, chromosome division was not performed normally, chromosomes was in two pair state. At this study microtubules and spindle pole body were observed in the nucleus of the cells not given high pressure stress, but localization of tubulin was observed along the microtubules by high pressure. It was thought that it will be multiplied to start growth. When cells subjected to pressure treatment were observed in detail with an electron microscope, a number of cells influenced or disappeared from tubulin and spindle body which appeared at cell division were observed.

S. pombe, fission yeast close to higher organisms, showed a high susceptibility to high

pressure as cell viability dramatically decreased above 100 MPa. In this experiment using *S. pombe JY 333* (*ade 6 - M 216 leu 1 h* $^{-}$), which is a laboratory strain, cells which were multiplied similarly to *S. cerevisiae* were obtained by high pressure, and from the separation ratio of genetic marker *ade* requirement. It turned out to be self-diploidization.

These results suggested that hydrostatic pressure was effective not only for brewing yeast such as baker's yeast but also as a breeding technology for higher organisms such as fish eggs. Fish eggs treated by high pressure treatment they were reported to get the fish with increased chromosome sets. This phenomenon was clarified in this research findings on the phenomenon that higher eukaryotes were possible for breeding (Dasgupta, S. 1962), (Onozato, H. 1984).

In the classical developing method of industrial strains such as baker's yeast and sake yeast haploid strains obtained by spores separated from parental strains having useful properties were crossed with haploid strains having another trait by a sexual joining method, and got stable diploid strains. Nakatomi et al developed efficient method for separating ascospores from the mixture of vegetative cells and ascospore using the difference of tolerance due to the high pressure of the vegetative cells and ascospores. These technology can be used for improve new kinds of industrial yeasts, lactic acid bacteria on behalf of physical and chemical mutation sources. On this method it is difficult to remove vegetative cells from sporulating cells and separating only spores were hard work involving skill and labor. This methods could be simply carried out spore separation.

Conventionally, mutant strains have been produced using physical methods such as UV irradiation and chemical substances such as NTG, EMS etc. However, these methods were not suitable for improving mutant strains because lacking practically useful genes were acquired in many cases. There was a possibility that industrial strains with other properties could be developed while maintaining useful properties such as freezing tolerance and high sugar dough performance in baker's yeast.

In general, industrial baker's yeast strains should have useful properties such as frozen dough

resistance and make rich flavor. For developing industrial strains, it is very important issue for retaining useful properties and eliminating bad property (low growth rate, bad dehydration efficiencies, and not resistant at high temperature around 37°C). This method can be improved to make tetraploid cells from diploid cells by hydrostatic pressure treatment without eliminating useful properties, and segregated from them and screening useful diploid strains. In fact, author could be successful to develop the new baker's yeast in a short period by using hydrostatic pressure method and it has been still used at many bakeries for 25 years in Japanese market.

Since this technology is easy to get mutants and self-polyploidy strains maintained some useful properties, I would like to expect that this technology will be popularized in the brewing and food industry as a useful developing method for microorganisms and higher eukaryotes.

Keywords: Hydrostatic pressure, high pressure, Yeast, Homozygous tetraploid, *Saccharomyces cerevisiae, Schizosaccharomyces. pombe,* Industrial yeast, Baker's yeast

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