Full Paper

A simple method for culture and stable maintenance of giant spheroplasts from the yeast *Saccharomyces cerevisiae*

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When spheroplasts of the yeast *Saccharomyces cerevisiae* are cultured in liquid medium containing osmotic stabilizer, they undergo nuclear division and growth without cell division, resulting in the formation of giant spheroplasts with multinuclei. In this study, we report a simple method for the culture and stable maintenance of giant spheroplasts. The selection of culture media and cell concentration was found to be important for the growth and maintenance of giant spheroplasts. Among the conditions that we tested, static culture in a synthetic Burkholder's medium in 96-well U-bottomed culture plates was most effective. Under appropriate conditions, we could maintain giant spheroplasts for more than 6 days without proliferation of whole cells or marked lysis. The average diameter of spheroplasts can vary from 16 to 53 μm , depending on their initial concentration.

Key Words——DAPI; 2-deoxyglucose; giant spheroplast; mitochondrial nucleoids; *Saccharomyces cerevisiae*; yeast

Introduction

In the past three decades, several methods have been reported for the growth of yeast spheroplasts in liquid culture. When spheroplasts of *Saccharomyces cerevisiae* were inoculated in rich medium containing 0.4 M KCl as an osmotic stabilizer and cultured for 7–8 h with occasional manual shaking, they entered the first round of mitosis at low cell density. Spheroplasts inoculated at higher cell density showed significant retardation of mitosis (Doi and Doi, 1979, 1982). Doi and Doi (1982) further showed that spheroplasts underwent the first round of DNA synthesis at roughly

the same time as normal cells, but underwent the first round of nuclear division significantly later than normal cells. Spheroplasts cultured in YPG (1% yeast extract, 2% peptone, 2% glucose) medium supplemented with 1 M sorbitol and zymolyase for 18 h increased their diameter threefold, resulting in giant spheroplasts with an average diameter of 13.4 μm (Tamai et al., 1983). Our previous study showed that mitotic nuclear division could be visualized more clearly in giant spheroplasts than in whole cells by DAPI staining and immunofluorescence microscopy using anti-tubulin antibody (Sando et al., 1987; Miyakawa, 2000).

The formation of giant spheroplasts of microbial cells overcomes the technical problems that are otherwise encountered owing to their small size. Giant spheroplasts of *E. coli* were successfully generated by spheroplast incubation methods (SI methods) to measure membrane potentials of plasma membrane by a patch-clamp method (Kuroda et al., 1998). Yabe et al.

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(1999) applied the SI methods to generate giant spheroplasts from yeast cells. They produced giant spheroplasts with an average diameter of 20 μm by incubating spheroplasts in the presence of 2-deoxyglucose (2-DG) to inhibit cell wall synthesis. Giant vacuoles were isolated from giant spheroplasts by osmotic shock. They succeeded for the first time in measuring membrane potentials of vacuole membranes by a patch-clamp method. This method opened the way to directly measure the membrane potentials of vacuoles of yeast cells that expressed the two-pore- K^+ channel (NtTPK1) of tobacco tonoplasts (Hamamoto et al., 2008).

Enlargement of yeast cells will lead to the development of new technology to enable yeast cells to be handled like plant or animal cells. However, protocols for the generation of giant spheroplasts from yeast cells are complicated and differ among studies. To widely use giant spheroplasts for many purposes, it is necessary to develop simple methods for their culture and stable maintenance. In our experiences, two problems must be overcome for this purpose: (1) regeneration of whole cells and (2) lysis of giant spheroplasts during spheroplast culture. In this study, we demonstrate that the static culture of spheroplasts in a synthetic Burkholder's medium with 96-well U-bottomed culture plates is very effective for both enlargement and stable maintenance of giant spheroplasts.

Materials and Methods

Strains and culture. *Saccharomyces cerevisiae* G2-2 diploid strain (*Mat a/ α*) was used (Miyakawa et al., 1987). Cells were aerobically precultured for 30 h at 30°C to stationary phase in YPD (1% yeast extract, 2% peptone, 2% glucose) medium or in a synthetic Burkholder's medium using a Sakaguchi flask with reciprocal shaking. Burkholder's medium contained the following ingredients per 1 liter: glucose 20.0 g, succinic acid 2.95 g, KH_2PO_4 1.5 g, $(\text{NH}_4)_2\text{SO}_4$ 2.0 g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.33 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.04 mg, H_3BO_3 0.6 mg, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.04 mg, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.31 mg, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.25 mg, KI 0.1 mg, Na_2MoO_4 0.025 mg, biotin 0.002 mg, Ca-pantothenate 0.2 mg, inositol 10.0 mg, nicotinic acid 0.2 mg, pyridoxine HCl 10.2 mg, and thiamine HCl 10.2 mg, pH 5.0.

Spheroplast formation. Cells at the stationary phase were collected and pretreated in solution A

(0.8 M sorbitol, 25 mM K-phosphate buffer pH 7.5, 0.2 M β -mercaptoethanol) for 20 min at 30°C at a cell concentration of 3×10^7 cells/ml. After being washed with solution A, they were treated with zymolyase 20T (Seikagaku Kogyo Co., Ltd., Tokyo, Japan) at a final concentration of 400 $\mu\text{g}/\text{ml}$ for 60 min at 30°C in solution A at a cell concentration of 3×10^7 cells/ml. Complete spheroplasting was checked by phase-contrast microscopy. Spheroplasts were twice washed with solution A and suspended in a small volume of solution A.

Culture of giant spheroplasts. Spheroplasts were cultured in five different culture media as follows: (1) YPD-sorbitol-zymolyase (YPDSZ) medium, YPD medium adjusted to pH 7.5 with NaOH and supplemented with 0.8 M sorbitol and 100 $\mu\text{g}/\text{ml}$ zymolyase 20T; (2) SD-sorbitol-zymolyase (SDSZ) medium, SD medium (0.67% yeast nitrogen base, 2% glucose) adjusted to pH 7.5 with NaOH and supplemented with 0.8 M sorbitol and 100 $\mu\text{g}/\text{ml}$ zymolyase 20T; (3) SD-sorbitol-zymolyase-casamino acid (SDSZC) medium, SDSZ medium supplemented with 1% casamino acid (vitamin-free; Difco, MI, USA); (4) Burkholder-sorbitol-zymolyase (BSZ) medium, Burkholder's medium adjusted to pH 7.5 with NaOH and supplemented with 0.8 M sorbitol and 100 $\mu\text{g}/\text{ml}$ zymolyase 20T; (5) Burkholder-sorbitol-zymolyase-casamino acid (BSZC) medium, BSZ medium supplemented with 1% casamino acid (vitamin-free; Difco).

Each 200 μl of culture medium was poured in wells of 96-well U-bottomed culture plates. Spheroplasts were inoculated in culture medium at a cell concentration of 1×10^5 – 5×10^6 cells/ml and statically cultured in a moist chamber at 30°C. In order to elucidate the effect of 2-deoxyglucose (2-DG) on the growth of giant spheroplasts, 2-DG was added to each well from 120 mM stock solution at 1.5 mM to 12 mM. As control experiments, spheroplasts were cultured in 100 ml of BSZ medium in a 100 ml Erlenmeyer flask at 30°C with gentle stirring.

Observation of giant spheroplasts. Giant spheroplasts were observed using Thoma's hemocytometer with a differential interference contrast (DIC) microscope (Axio Imager, Carl Zeiss), or a fluorescence and phase contrast microscope (BHS-RFK, Olympus). As the thickness of Thoma's hemocytometer is 100 μm , giant spheroplasts with a diameter of less than 100 μm can be observed without being squashed by the glass cover. The cell concentration of spheroplasts was de-

terminated using Thoma's hemocytometer. The diameter of 50 spheroplasts was measured for each sample with Thoma's hemocytometer using ocular micrometers.

For the staining of cells with 4',6-diamidino-2-phenylindole (DAPI), whole cells and giant spheroplasts were fixed with 5% glutaraldehyde for 1 h at room temperature and washed twice with NS buffer (20 mM Tris-HCl, pH 7.6, 0.25 M sucrose, 1 mM EDTA, 1 mM MgCl₂, 0.1 mM ZnSO₄, 0.1 mM CaCl₂, 0.8 mM PMSF, and 0.05% 2-mercaptoethanol; Miyakawa et al., 1987). Samples were stained with DAPI (1 µg/ml) dissolved in NS buffer on a glass slide.

Results

Effect of culture media on spheroplast culture

Cells precultured to stationary phase in YPD or Burkholder's medium were converted to spheroplasts, which were inoculated at 1×10^6 cells/ml in five different media of YPDSZ, SDSZ, SDSZC, BSZ and BSZC in a 96-well culture plate. When spheroplasts were generated from cells grown in YPD medium, whole cells reproducibly regenerated and rapidly grew in all of the five media within 72 h of culture, although we had checked by microscopy in each experiment that spheroplast formation was complete (data not shown). When spheroplasts generated from cells grown in Burkholder's medium were cultured in YPDSZ medium, regeneration of whole cells and lysis of spheroplasts were also discerned after 72 h (Fig. 1A). Although regeneration of whole cells was not marked in SDSZ or SDSZC media compared with that in YPDSZ culture, lysis of spheroplasts was clear after 72 h of culture in these two media (Fig. 1B and 1C). In contrast, regeneration of whole cells did not occur in BSZ or BSZC media during 72 h of culture (Fig. 1D and 1E).

In a control experiment, spheroplasts generated from cells grown in Burkholder's medium were cultured in BSZ medium in 100 ml Erlenmeyer flasks with gentle stirring using a magnetic stirrer at 30°C instead of 96-well culture plates. However, in these cultures, whole cells regenerated and rapidly proliferated without exception during 36 h–72 h of culture in all culture media tested. Marked lysis of giant spheroplasts also occurred (data not shown).

These results demonstrated that preculture of whole cells in Burkholder's medium and subsequent culture of the spheroplasts in BSZ or BSZC medium with the

Fig. 1. Phase contrast microscopy of giant spheroplasts cultured in five different media.

Cells were precultured in Burkholder's medium to stationary phase, then treated with zymolyase to generate spheroplasts. Spheroplasts were cultured in 200 µl of culture medium in 96-well U-bottomed culture plates at an initial cell concentration of 1×10^6 cells/ml for 72 h at 30°C. A, YPDSZ medium; B, SDSZ medium; C, SDSZC medium; D, BSZ medium; E, BSZC medium. Bar represents 50 µm. An arrow shows a whole cell that regenerated during culture.

use of 96-well culture plates were effective for cultivation of spheroplasts to minimize the proliferation of whole cells and lysis of spheroplasts.

Effects of spheroplast concentration on growth and stability of giant spheroplasts

The effect of initial spheroplast concentration on the growth and stability of giant spheroplasts was investigated in BSZ culture. A representative experiment is shown in Fig. 2. When spheroplasts were inoculated at 5×10^5 cells/ml, 1×10^6 cells/ml and 5×10^6 cells/ml, their average diameter increased during the first 30 h of culture and reached 30 µm, 26 µm and 16 µm after 48 h of culture, respectively (Fig. 2A). Thereafter, the increase of the average diameter of giant spheroplasts was very slow up until 144 h of culture. In contrast, when spheroplasts were inoculated at 1×10^5 cells/ml, spheroplasts continued to grow during 96 h of culture

Fig. 2. The effect of initial cell concentration on cell size and maintenance of giant spheroplasts.

Spheroplasts were cultured in 200 μl of BSZ medium at four different cell concentrations: $\circ$ 1×10^5 cells/ml, $\bullet$ 5×10^5 cells/ml, $\square$ 1×10^6 cells/ml, $\blacksquare$ 5×10^6 cells/ml. A, Changes in average diameters of giant spheroplasts. Bars are standard deviations; B, changes in cell concentrations of giant spheroplasts. The size and cell concentration of spheroplasts that were inoculated at 1×10^5 cells/ml could not be measured owing to lysis of spheroplasts after 96 h of culture.

and the diameter of the giant spheroplasts finally reached 53 μm on average. We could not measure the average diameter of giant spheroplasts owing to the marked lysis of spheroplasts after 96 h of culture. When spheroplasts were inoculated at 1×10^6 cells/ml and 5×10^6 cells/ml, about 60% of them were stably maintained after 144 h of culture (Fig. 2B). When spheroplasts were inoculated at a cell concentration of 5×10^5 cells/ml, about 30% of the giant spheroplasts survived after 144 h of culture. When spheroplasts were inoculated at 1×10^5 cells/ml, significant lysis of spheroplasts occurred and less than 10% of giant spheroplasts remained after 96 h of culture (Fig. 2B). These results showed that spheroplasts are stable when they are inoculated at cell concentrations of more than 5×10^5 cells/ml. Under these culture conditions, regeneration of whole cells did not occur.

Effects of 2-DG on growth of giant spheroplasts

Yabe et al. (1999) reported that the addition of 3 mM (0.05%, w/w) 2-DG, which corresponds to a ratio of one molecule of 2-DG to 40 molecules of glucose in the culture, greatly facilitated the formation of giant spheroplasts. To ascertain the effect of 2-DG, we investigated the diameter of giant spheroplasts cultured in BSZ medium at an initial cell concentration of 1×10^5 cells/ml. When spheroplasts were cultured without 2-DG or with 1.5 mM, 2.0 mM, 3.0 mM, 6.0 mM or 12 mM 2-DG, the diameter of giant spheroplasts became smaller as the concentration of 2DG became

Fig. 3. The effect of 2-DG on growth of giant spheroplasts.

Spheroplasts were cultured in 200 μl of BSZ medium at a cell concentration of 1×10^5 cells/ml in the presence of six different concentrations of 2-DG: $\circ$ without 2-DG, $\bullet$ 1.5 mM, $\square$ 2.0 mM, $\blacksquare$ 3.0 mM, $\triangle$ 6.0 mM and $\blacktriangle$ 12 mM. Changes in average diameters of giant spheroplasts during culture are shown. Bars are standard deviations.

higher (Fig. 3). Whereas the diameter of spheroplasts without 2DG was 44 μm after 48 h of culture, that of spheroplasts was 37, 30, 24, 21 and 21 μm in the presence of 1.5, 2.0, 3.0, 6.0 and 12 mM 2-DG, respectively. These results indicated that 2-DG inhibits the growth of giant spheroplasts, even at a concentration of 3 mM.

Morphology of giant spheroplasts

Giant spheroplasts had one or more giant vacuoles surrounded by a layer of cytoplasm (Fig. 1, Fig. 4). As shown in Fig. 4B and 4C, the ratio of cytoplasm to vacuole in giant spheroplasts was affected by the initial cell concentration. When the initial cell concentration was 1×10^5 cells/ml, the layer of cytoplasm was relatively thick (Fig. 4B). On the other hand, when cells were inoculated at 5×10^5 cells/ml or 1×10^6 cells/ml, spheroplasts had a large vacuole and a thin layer of cytoplasm (Fig. 4C, Fig. 1D). Extraordinarily giant spheroplasts with a diameter of more than $80 \mu\text{m}$ were found in cultures with very low frequency, when spheroplasts were inoculated at 1×10^5 cells/ml or 5×10^5 cells/ml (Fig. 4D). Whole cells and giant spheroplasts were fixed with 5% glutaraldehyde, slightly squashed, and stained with DAPI (Fig. 4E and 4F). Whole cells cultured in Burkholder's medium harbored

50–60 mitochondrial nucleoids (Miyakawa et al., 1987). However, it was difficult to observe all the mt-nucleoids on the same focal plane because of their rather rigid cell wall (Fig. 4E). On the other hand, giant spheroplasts were easy to squash and more than 400 of the mt-nucleoids were visible on the same focal plane (Fig. 4F).

Discussion

As we and other researchers described previously, spheroplasts increase their volume during culture in a medium supplemented with osmotic stabilizer. Nuclear division proceeds in giant spheroplasts. However, long-term culture of spheroplasts has not been performed. This is due to the proliferation of whole cells and lysis of giant spheroplasts during culture. Once whole cells regenerate, they rapidly proliferate and re-

Fig. 4. Morphology of giant spheroplasts.

A–D, DIC images; A, whole cells; B, giant spheroplasts that were cultured in BSZ medium for 72 h at an initial cell concentration of 1×10^5 cells/ml; C, giant spheroplasts that were cultured in BSZ medium for 2 weeks at an initial cell concentration of 5×10^5 cells/ml; D, giant spheroplasts with a diameter of $87 \mu\text{m}$ that were cultured in BSZ medium for 2 weeks at an initial cell concentration of 5×10^5 cells/ml; A–D have the same scale. Bar in Fig. 4D represents $50 \mu\text{m}$; E and F, DAPI-staining images; E, a whole cell grown in Burkholder's medium to stationary phase; F, a giant spheroplast grown in BSZ medium for 2 weeks at a cell concentration of 1×10^6 cells/ml. Cells were fixed with 5% glutaraldehyde, lightly squashed and stained with DAPI. E and F have the same scale. Bar in Fig. 4F represents $10 \mu\text{m}$.

place spheroplasts during culture. Therefore, the use of 0.05% 2-DG, a cell wall inhibitor was found to be critical for the production of giant spheroplasts (Yabe et al., 1999).

We found several important conditions for the culture and stable maintenance of giant spheroplasts in this study. First, the preculture medium of whole cells significantly affects the frequency of regeneration of whole cells during spheroplast culture. In this study, the use of Burkholder's medium significantly reduced regeneration of whole cells during spheroplast culture. Second, the use of BSZ medium for spheroplast culture was also effective to minimize the regeneration of whole cells. These results demonstrate that the use of synthetic medium is much better than the use of nutrient-rich YPD medium for the culture of giant spheroplasts. Limitation of nutrient in a synthetic medium might facilitate growth of spheroplasts without regeneration of whole cells. Third, static culture in a 96-well culture plate greatly improved the maintenance of giant spheroplasts during long-term culture. This method enabled us to culture giant spheroplasts even for 2 weeks without regeneration of whole cells or significant lysis of spheroplasts. Fourth, we can omit the addition of 2-DG that inhibits the growth of giant spheroplasts (Fig. 3).

We can rapidly examine the effects of various concentrations of reagents such as 2-DG on the growth of spheroplasts in a 96-well culture plate. The cell number in a 200- μ l culture medium is sufficient for observation of cells by microscopy. The size of spheroplasts and the ratio of cytoplasm to vacuole can be changed by altering the initial cell concentration of spheroplasts. This method is simple and can be widely applied for the observation of organelles such as mitochondria, as well as the measurement of membrane potential.

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References

- Doi, A. and Doi, K. (1979) Mitotic behavior of yeast spheroplasts in liquid culture. *Cell Struct. Funct.*, **4**, 261–266.
- Doi, A. and Doi, K. (1982) Progress in the mitotic cycle of yeast spheroplasts in liquid culture. *Exp. Cell Res.*, **137**, 95–110.
- Hamamoto, S., Marui, J., Matsuoka, K., Higashi, K., Igarashi, K., Nakagawa, T., Kuroda, T., Mori, Y., Murata, Y., Nakanishi, Y., Maeshima, M., and Yabe, I. (2008) Characterization of a tobacco TPK-type K^+ channel as a novel tonoplast K^+ channel using yeast tonoplasts. *J. Biol. Chem.*, **25**, 1911–1920.
- Kuroda, T., Okuda, N., Saitoh, N., Hiyama, T., Terasaki, Y., Anazawa, H., Hirata, A., Mogi, T., Kusaka, I., Tsuchiya, T., and Yabe, I. (1998) Patch clamp studies on ion pumps of the cytoplasmic membrane of *Escherichia coli*. *J. Biol. Chem.*, **273**, 16897–16904.
- Miyakawa, I. (2000) Immunofluorescence microscopy of dividing nuclei in yeast, *Saccharomyces cerevisiae*. *Cytologia*, **65** (2), Technical note.
- Miyakawa, I., Sando, N., Kawano, S., Nakamura, S., and Kuroiwa, T. (1987) Isolation of morphologically intact mitochondrial nucleoids from the yeast, *Saccharomyces cerevisiae*. *J. Cell Sci.*, **8**, 431–439.
- Sando, N., Mori, K., Miyakawa, I., and Kuroiwa, T. (1987) Nuclear division of protoplasts of *Saccharomyces cerevisiae*. *Yeast Genetics and Molecular Biology News JAPAN*, **20**, 57 (in Japanese).
- Tamai, Y., Shinmoto, H., and Takakuwa, M. (1983) Preparation and some properties of giant-protoplasts from *Saccharomyces cerevisiae*. *Nippon Nogeikagaku Kaishi*, **57**, 667–669 (in Japanese).
- Yabe, I., Horiuchi, K., Nakahara, K., Hiyama, T., Yamanaka, T., Wang, P.-C., Toda, K., Hirata, A., Ohsumi, Y., Hirata, R., Anraku, Y., and Kusaka, I. (1999) Patch clamp studies on V-type ATPase of vacuolar membrane of haploid *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **274**, 34903–34910.