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Mass Production of Yeast Spores from Compressed Yeast

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Abstract *Saccharomyces* yeast spores are more resistant to drying and storage than vegetative cells. For the mass production of yeast spores, compressed yeast was directly inoculated into a sporulation medium (SM). The effects of inoculum size and the addition of rice wine cake (RWC) into SM on the sporulation were examined using flasks. With 1% inoculum of compressed yeast, 1.45×10^8/ml of asci was obtained. The addition of 0.5% RWC into SM improved the cell growth and spore yield, and the number of asci formed was 2.31×10^8/ml. The effects of culture temperature, temperature-shift, and concentrations of inoculum, potassium acetate, and RWC on the sporulation were also evaluated using a jar fermentor. The optimum temperature for spore formation was 22°C where the number of asci formed was 2.46×10^8/ml. The shift of culture temperature from initial 30°C for 1 day to 22°C for 3 days increased the number of asci formed to 2.96×10^8/ml. The use of 2% (w/v) inoculum of compressed yeast, 2% potassium acetate, and 1% (w/v) RWC in SM with the shift of culture temperature of initial 30°C to 22°C resulted in 90% sporulation ratio and formation of 6.18×10^8 asci/ml.

Key words: Yeast spores, *Saccharomyces*, compressed yeast, sporulation medium, rice wine cake, potassium acetate, shift of culture temperature.

Dried yeast cells are the more preferred form for long storage and distribution than the perishable wet yeast cells. However, most vegetative cells die during the drying processes [7], and even the survivors eventually die after a long storage at room temperature [6]. Yeast spores are more resistant to many environmental stresses than vegetative cells [11, 14], and they are also useful as a biocatalyst. The use of immobilized yeast spores in a bioreactor system has been reported for the production of valuable chemicals [12].

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Sporulation of yeast cells is composed of two stages of culture; the vegetative cells are grown in a presporulation medium (PSM) and then transferred to a sporulation medium (SM) to induce sporulation [2, 4, 11]. The PSM is a nutrient-rich medium and the SM is a starvation medium containing a nonfermentative carbon source such as acetate but lacking nitrogen and fermentable carbon sources [3]. The culture of yeast cells in a presporulation medium requires 2 days of cultivation. Compressed yeast is the mass of live cells of Baker's yeast and is easily purchased in the market. If compressed yeasts are used for the production of yeast spores, the time and efforts to grow the vegetative cells in PSM and to harvest the cell mass could be saved.

Most sporulations of yeast have been conducted in a small quantity on agar plates or in shaking flasks, and the spores are used for genetic studies or strain improvement via hybridization [1, 2, 11]. However, mass production of yeast spores has not yet been reported. The present investigation was conducted to examine the culture conditions for the mass production of maximal number of spores directly from compressed Baker's yeast.

Materials and Methods

Yeast Strain

Compressed Baker's yeast (Cho Heung Chemical Co., Ltd., Korea) was used for the mass production of spores. *Saccharomyces* spp. FR was used to examine the survival ratio of vegetative cells and spores after drying and storage.

Media and Cell Culture

YPD medium containing 1% yeast extract (Y), 2% peptone (P), and 2% dextrose (D) was used to grow and maintain yeast cultures. YPD agar contained 1.5–2.0% agar. Yeast cells were grown at 30°C for 2 days.

Table 1. Survival ratio of vegetative cell and spore[a,b] after vacuum drying[c] and storage at different temperatures.

Cell/spore	Storage temperature (°C)	Survival ratio (%)		
		Right after drying	After 1 month	After 2 month
Vegetative cell	-	5	0	0
Spore	Room temperature	103[d]	75	52
Spore	30°C	103[d]	63	ND[e]
Spore	37°C	103[d]	51	ND

[a]One-hundred-sixty milliliters of yeast culture were inoculated into 1,600 ml of SM in a 2.5-l jar fermentor, and the fermentor was operated at 25°C with 1 vvm aeration and 200 rpm agitation for 4 days for sporulation.
[b]The sporulation ratio after 4 days was 60%.
[c]The yeast spore suspension was pelleted, and 0.5 g of pellet was dried at 63°C in a vacuum oven for 1.5 h and stored at different temperatures and for different periods.
[d]The viable cell number of dried spore pellets was 2.1×10^{10} CFU/g.
[e]ND, not determined.

Sporulation Medium (SM) and Culture

The SM (pH 6.5) was 1% potassium acetate. When necessary, 0.5% rice wine cake (RWC) was added into the SM. RWC is the filtered solid waste from rice wine fermentation. According to the analysis of the Scientec Lab. Center Co., Ltd. (Daejeon, Korea), the RWC contains 56.78% water, 34.03% carbohydrate, 5.84% protein, 0.62% lipid, 2.38% cellulose, 0.35% minerals, 2.08 ppm vitamin B1, 1.39 ppm vitamin B2, 407.45 ppm vitamin C, 3×10^9/g of total yeast cells, and 4.1×10^3/g of viable cells. Five-hundred milliliters of flask containing 100 ml of SM were incubated at 25°C with 200 rpm in a rotary shaking incubator.

Fermentor Culture

A jar fermentor with 2.5 l capacity, equipped with an automatic temperature control system (Korea fermentor, Korea), was used. The fermentor with the working volume of 1.6 l was operated with 200 rpm agitation and 1 vvm aeration. Since the preliminary experimental results showed that the sporulation ratio was rapidly increased after 3 days and reached a maximum after 4 days, the fermentor culture for sporulation was conducted for 3 and 4 days.

Survival Ratio of Vegetative Cell and Spore

Half a gram of cell or spore pellet placed on a 5-cm aluminum pan was dried in a 63°C vacuum oven for 1.5 h [7]. The vacuum drying was carried out using a freeze dryer (EYELA Model FD-1, Japan). The cell and/or spore pellet was placed into 30 ml of distilled water (D.W.), rehydrated in a 40–43°C water bath for 10 min, and voltexed to make cell/spore suspension. The viability count of cell/spore suspension was determined by the spreading plate method using YPD agar plate. The survival ratio was expressed by the percentage of viable cell number after drying and/or storage versus viable cell number before drying and/or storage.

Measurement of Cell Number and Sporulation Ratio

The number of cells and asci containing spores were counted with a hemacytometer under a microscope. Only the asci containing a set of 4 spores (tetrad) were counted. The sporulation ratio (%) was calculated as the percentage of asci per total number of cells and asci.

Results and Discussion

Survival Ratio of Spore After Drying and Storage

Table 1 shows the survival ratio of spore after vacuum drying and storage at different temperatures. The survival ratio of spores was clearly much higher than that of the vegetative cells after vacuum drying: Only 5% of the vegetative cells survived after the drying, and the survival ratio of spores after vacuum drying was more than 100%, which seemed to be due to increased colony forming units, resulting from the rupture of the ascus sac during the

Table 2. Effect of inoculum size on the sporulation[a].

Inoculum size (%, w/v)	3 days				4 days			
	pH	Cell no. ($\times10^8$/ml)	Ascus no. ($\times10^8$/ml)	Sporulation ratio (%)	pH	Cell no. ($\times10^8$/ml)	Ascus no. ($\times10^8$/ml)	Sporulation ratio (%)
0.1	9.04	1.48	0.52	35	9.13	1.05	0.49	47
1.0	9.10	3.92	1.10	28	9.28	3.60	1.45	40
10.0	8.22	17.04	0.00	0	8.63	16.00	0.00	0

[a]The SM, composed of 1% potassium acetate, was inoculated with different amounts of compressed yeast and incubated at 25°C for 3 and 4 days in a rotary shaking incubator.

Table 3. Effect of different concentrations of rice wine cake added into SM on the cell growth and sporulation[a].

RWC (%, w/v)	3 days				4 days			
	pH	Cell no. ($\times10^8$/ml)	Ascus no. ($\times10^8$/ml)	Sporulation ratio (%)	pH	Cell no. ($\times10^8$/ml)	Ascus no. ($\times10^8$/ml)	Sporulation ratio (%)
0.0	9.42	3.10	0.95	30.5	9.61	3.27	1.37	42.0
0.5	9.40	3.21	1.66	51.8	9.45	3.30	2.31	70.0
1.0	9.28	3.39	1.29	38.2	9.38	3.32	1.83	55.0
2.0	9.04	4.64	1.07	23.0	9.17	4.52	1.85	41.0

[a]The SM (100 ml) containing different amounts of RWC was inoculated with 1.0 g of compressed yeast and incubated at 25°C for 3 and 4 days in a rotary shaking incubator.

vacuum drying and/or rehydration of the ascus and the subsequent release of the tetrad spores from the ascus sac. The lower storage temperature showed a higher survival ratio of spore. After 2 months of storage at room temperature, half the amount of the initial dried spores were still alive. These results indicate that yeast spores are much more superior to the vegetative cells in survival ratio after vacuum drying, and that the dried spores can be preserved for a certain period of time even at room temperature without refrigeration.

Production of Spores in Flask

Effect of Inoculum Size. Table 2 shows the effect of inoculum size on the sporulation. Although the highest sporulation ratio was observed using 0.1% (w/v) inoculum, the maximal number of asci (1.45×10^8/ml) was obtained with 1.0% of inoculum. In another experiment, using inoculum sizes of 0.5–3.0%, 1.0% inoculum also showed the highest number of asci production (data not shown). Ten percent inoculum resulted in no spore formation at all: At high cell density, sporulation is much less, and the factors to limit sporulation of dense cell populations include the accumulation of CO_2 to inhibitory levels, decreased supply of oxygen, and exhaustion of substrate [5, 11]. Another noticeable contributing factor is pH change of the SM; the pH of the SM of 0.1 and 1% inoculum, where the sporulation occurred, was above 9, while that of 10% inoculum, where no sporulation occurred, was 8.63. This characteristic rise of medium pH from neutral to above 9 for the optimum sporulation was also observed in other works [5, 8, 13]. Ohkuni *et al.* [13] explained that the rise in pH is due to accumulation of bicarbonate as cells respire acetate and evolve CO_2, and that an alkaline medium inhibits proliferation of cells and stimulates meiosis and sporulation.

Effect of Addition of RWC into SM. RWC is the abundant waste in rice winery and can be used as the nutrient supplement for microbial growth. Previously, we have shown that the RWC could be used as a substrate to produce yeast cells and spores [8]. The RWC was added into SM to improve the yeast cell growth, sporulation ratio, and consequently asci number (Table 3). The best RWC concentration to obtain the highest sporulation ratio and asci number was 0.5%. The addition of 2.0% RWC produced the highest cell number but the lowest sporulation ratio, which resulted in a lower asci number than that with 0.5% RWC. These results indicated that the addition of a small amount of nutrient to SM increased the sporulation rate [11], but the higher concentration of nutrient acted as an inhibitory factor on the sporulation ratio [8].

Production of Spores in a Fermentor

Effect of Culture Temperature. The effect of culture temperature on the cell growth and sporulation was examined using a jar fermentor (Table 4). The optimum temperature for the sporulation and the highest number of asci was 22°C. It has earlier been reported that the cell growth at 30°C was higher than that at 22°C, but the sporulation rate of various Baker's yeasts was higher at 22°C than at 30°C [2]. The fact that the sporulation occurs at lower temperature [1, 2] could be related to the fact that

Table 4. Effect of culture temperature on cell growth and sporulation in a fermentor[a].

Temperature (°C)	3 days				4 days			
	pH	Cell no. ($\times10^8$/ml)	Ascus no. ($\times10^8$/ml)	Sporulation ratio (%)	pH	Cell no. ($\times10^8$/ml)	Ascus no. ($\times10^8$/ml)	Sporulation ratio (%)
18	9.42	2.65	1.01	38	10.68	2.60	1.09	42
22	9.10	2.70	1.40	52	9.52	3.90	2.46	63
26	10.70	3.90	1.60	41	9.36	4.20	2.14	51
30	9.04	4.40	0.75	17	9.47	4.60	0.92	20

[a]The SM, composed of 1% potassium acetate, and 0.5% RWC were inoculated with 1.0% compressed yeast (2.47×10^8/ml) and incubated at various temperatures for 3 and 4 days in a 2.5-l fermentor at 1 vvm aeration with 200 rpm agitation.

Table 5. Effect of temperature shift on cell growth and sporulation in a fermentor[a].

Temperature (°C)	3 days				4 days			
	pH	Cell no. ($\times10^8$/ml)	Ascus no. ($\times10^8$/ml)	Sporulation ratio (%)	pH	Cell no. ($\times10^8$/ml)	Ascus no. ($\times10^8$/ml)	Sporulation ratio (%)
22	9.10	2.70	1.40	52	9.52	3.90	2.46	63
30→22	9.38	3.90	1.87	48	9.67	4.05	2.96	73

[a]A jar fermentor, containing SM of 1% potassium acetate and 0.5% RWC, was inoculated with 1% compressed yeast of 2.47×10^8/ml and operated initially at 30°C for 1 day and then at 22°C for 2 or 3 days, totally 3 or 4 days, respectively.

mitochondrial protein synthesis in yeast cells favors a lower temperature [9, 10, 11]. The yeast sporulates only when mitochondria respire [9].

Effect of Temperature Shift. From the above results, it was realized that the highest cell number as well as the highest sporulation ratio were important to obtain the highest asci number.

If the cell initially multiplies highly at 30°C and later the temperature is shifted to 22°C for sporulation, then a higher number of asci might be obtained. Therefore, the effect of temperature shift on the cell growth and sporulation was examined, and the results are shown in Table 5. In this experiment, cells were incubated for 1 day at 30°C and incubated further for 3 days at 22°C. After 3 days, the cell number of the culture with the temperature shift from 30°C to 22°C was much higher than that of 22°C culture, however, the sporulation ratio of the culture with the temperature shift was slightly lower than that of 22°C culture. After 4 days, the temperature shift resulted in a higher cell number and sporulation ratio than those of the fixed temperature of 22°C. Finally, the number of asci formed was increased to 2.96×10^8/ml by the temperature shift from 2.46×10^8/ml of the fixed temperature.

Effect of Concentrations of Inoculum, Potassium Acetate, and Rice Wine Cake

It has previously been shown that the sporulation ratio is significantly decreased at high cell density (Table 2) [5, 11]. The optimal cell density to obtain an abundant population of asci was reported to be 10^7/ml [11], and the concentration of potassium acetate used for sporulation was 0.5 [2] or 1% [8, 11]. To obtain a final high asci number, sporulation at high cell density appears to be important. Therefore, increased size of inoculum to obtain a higher cell number was attempted. In this experiment, the concentrations of potassium acetate and RWC in SM were also increased and the temperature was shifted from initial 30°C for 1 day to 22°C for 3 days. As shown in Table 6, the highest sporulation ratio of 90% and ascus number of 6.18×10^8/ml were obtained by using 2.0% inoculum, 2.0% potassium acetate, and 1.0% RWC (Table 6). Further increase of the inoculum, potassium acetate, and RWC resulted in a much lower sporulation ratio and ascus number.

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References

1. Bilinski, C. A., I. Russell, and G. G. Stewart. 1986. Analysis of sporulation in brewer's yeast: Induction of tetrad formation. *J. Inst. Brew.* **92:** 594–598.
2. Codon, A. C., J. M. Gasint-Rairez, and T. Benitez. 1995. Factors which affect the frequency of sporulation and tetrad formation in *Saccharomyces cerevisiae* Baker's yeast. *Appl. Environ. Micobiol.* **61:** 630–638.
3. Esposito, R. E. and S. Klapholz, S. 1981. Meiosis and ascospore development, pp. 211–287. *In* J. N. Strathern, E.

Table 6. Effect of concentrations of inoculum, potassium acetate, and rice wine cake on cell growth and sporulation in a fermentor[a].

Concentration (%)			3 days				4 days			
Inoculum	KOAc[b]	RWC	pH	Cell no. ($\times10^8$/ml)	Ascus no. ($\times10^8$/ml)	Sporulation ratio (%)	pH	Cell no. ($\times10^8$/ml)	Ascus no. ($\times10^8$/ml)	Sporulation ratio (%)
1.0	1.0	0.5	9.38	3.90	1.87	48	9.50	4.05	2.96	73
2.0	2.0	1.0	9.51	7.43	4.01	54	9.87	6.87	6.18	90
3.0	3.0	1.5	9.74	9.42	2.26	24	9.97	9.35	2.90	31

[a]The SM was inoculated with different concentrations of compressed yeast and incubated at 30°C. After 1 day, the culture temperature was shifted to 22°C and incubated for another 2 and 3 days.
[b]KOAc, potassium acetate.

W. Jones, and J. R. Broach (eds.), *The Molecular Biology of the Yeast Saccharomyces Life Cycle and Inheritance.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, U.S.A.

4. Fast, D. 1973. Sporulation synchrony of *Saccharomyces cerevisiae* grown in various carbon sources. *J. Bacteriol.* **116:** 925–930.
5. Fowell, R. R. 1969. Sporulation and hybridization of yeasts, pp. 303–383. *In* A. H. Rose and J. S. Harrison (eds.), *The Yeasts*, vol. **1**. Academic Press, London.
6. Herrera, T., W. H. Peterson, E. J. Cooper, and H. J. Peppler. 1956. Loss of cell constituents on reconstruction of active dry yeast. *Arch. Biochem. Biophy.* **63:** 131–143.
7. Kim, K. and J. Y. Kim. 1999. Yeast cell cultivation to produce active dry yeast with improved viability. *Kor. J. Biotechnol. Bioeng.* **14:** 561–565.
8. Lim, Y. S., S. M. Bae, and K. Kim. 2004. Production of yeast spores from rice wine cake. *Kor. J. Microbiol. Biotechnol.* **32:** 184–189.
9. Marmirol, N., M. Ferri, and P. P. Puglisi. 1983. Involvement of mitochondrial protein synthesis in sporulation: Effect of erythromycin on macromolecular synthesis, meiosis, and ascospore formation in *Saccharomyces cerevisiae. J. Bacteriol.* **154:** 118–129.
10. Marmiroli, N. and T. Lodi. 1984. Modification of nuclear gene expression by inhibition of mitochondrial translation during sporulation in MATα/MATa diploids of *Saccharomyces cerevisiae. Mol. Gen. Genet.* **198:** 69–74.
11. Miller, J. J. 1989. Sporulation in *Saccharomyces cerevisiae*, pp. 489–541. *In* A. H. Rose and J. S. Harrison (eds.), *The Yeasts*, vol. **3**. Academic Press, New York, U.S.A.
12. Murata, K. 1993. Use of microbial spores as a biocatalyst. *Crit. Rev. Biotechnol.* **13:** 173–193.
13. Ohkuni, K., M. Hayashi, and I. Yamashita. 1998. Bicarbonate-mediated social communication stimulates meiosis and sporulation of *Saccharomyces cerevisiae. Yeast* **14:** 623–631.
14. Yonemoto, Y., T. Yamashita, M. Muraji, and W. Tatebe. 1993. Resistance of yeast and bacterial spores to high voltage electric pulses. *J. Ferment. Bioeng.* **75:** 99–102.