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A Modified Process for Producing High Quantities of Bio-Germanium in Yeast and a Study of Its Oral Toxicity

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Abstract Since germanium has been shown to be beneficial for the treatment of diseases such as cancer and rheumatic arthritis, we developed an adapted process of bio-germanium preparation using inorganic germanium. In the present study we determined the optimal conditions for culturing yeast *Saccharomyces cerevisiae* (KCTC-1199), and the best concentrations of inorganic germanium for the adaptation process. The resulting method was successful at producing high quantities of germanium yeasts. The following are the culture conditions that obtained the highest level of productivity: an inorganic germanium concentration of 3,000-5,000 ppm, a pH of 6.5, a temperature of 35°C, and 20 hr of incubation time. In addition to this high-yield quantity study, we observed the acute oral toxicity of mice treated with Geranti Bio-Ge Yeast®. We found no changes in body weight, or in the mortality between the control groups and the bio-germanium yeast group. There were also no digestive problems such as diarrhea that occurred in either group.

Keywords: *Saccharomyces cerevisiae*, inorganic germanium, adaptation process, bio-germanium

Introduction

In the early 20th century, water from a well located in Lourdes, France was known to be very effective at treating various diseases. It was later discovered that the water contained a high level of germanium and since that time many researchers have focused on studying its pharmacological effects. It has been shown that germanium is beneficial for certain diseases (1) such as cancer, rheumatic arthritis, and aging. Germanium elicits these benefits by increasing the generation of oxygen in cells (2); excluding heavy metals from cells (3); providing analgesic effects (1); and increasing immune effects through the induction of interferones, macrophages, and NK cells (4).

Organic germanium is safe and has excellent medical effects, while inorganic germanium is toxic to the kidneys and liver (5). Therefore, the production of organic germanium is considered to be important. It has been reported that germanium accumulates in the cells of certain microorganisms (6) where it binds to nucleic acids and proteins (7). Thus, microorganism are a means for producing high concentrations of organic germanium within short amounts of time, using biological methods that are environmental friendly and reaction conditions that are less severe (8).

After VanDyke *et al.* (9) reported that it was possible to produce organic germanium in yeasts (bio-germanium), the method became popular and has since been used for brewing alcohol and making bread; the yeast can also serve as recombinant hosts that express and secrete foreign proteins (10, 11).

In this study we examined a production process for generating high concentrations of bio-germanium using yeasts. This was done by determining the optimal conditions for culturing yeasts and the best concentrations of inorganic germanium for the adaptation process. We tested the optimal culture conditions for generating high amounts of bio-germanium based on nutrients, pH, temperature, and incubation time. Finally, we tested the acute oral toxicity of the bio-germanium yeasts in mouse model systems. Overall, these modified processes were very effective for generating high concentrations of bio-germanium yeasts, and for producing yeasts that were safe in animal models.

Materials and Methods

The production process for generating high quantities of bio-germanium in yeasts (Geranti Bio-Ge Yeast®) In this study, we used yeast *Saccharomyces cerevisiae* (KCTC-1199) acquired from KRIBB (Daejeon, Korea), and YM Broth (BD Bioscience, Franklin, NJ, USA) for the culture ground. All the nutrients were purchased from BD Bioscience and all the chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless mentioned.

The yeast was cultured in various nutrient broths that contained the following: 1, 2, 3, 4, and 5% glucose; 0.1, 0.2, 0.3, 0.4, and 0.5% peptone; 0.1, 0.2, 0.3, 0.4, and 0.5% yeast extract; malt extract; and water at 30°C for 48 hr.

The yeasts cultured in the above broths were collected by centrifugation. To produce bio-germanium yeast, a solution containing inorganic germanium (1,000-5,000 ppm) was mixed with concentrated yeasts (1:1 v/v ratio)

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and cultured at pH 6.0 at 30°C for 20 hr. In addition, a solution containing a higher concentration of inorganic germanium was selected and mixed with the concentrated yeasts at a 1:1 (v/v) ratio. This solution mixture was cultured at various pH levels and temperatures to find the optimal conditions for generating the highest quantity of germanium in the yeasts.

Measurement of bio-germanium in the yeast To quantify the amount of bio-germanium produced, the yeast culture solutions were concentrated and rinsed twice to remove any inorganic germanium on the yeasts' surface. The dried yeasts were heated and lysed in 30 mL HNO_3, which was then adjusted to a pH of 6.0. One mL of this solution was mixed with 1 mL phenylfluor and 1 mL cyclohexane, and incubated at 30°C for 20 hr. The absorbance was measured at 525 nm to determine the amount of bio-germanium in the yeasts.

A safety experiment testing the acute oral toxicity of Geranti Bio-Ge Yeast® in mice Female balb/c mice (5-6 weeks old) were purchased from Jackson Korea and kept at 24±2°C, 50-60% humidity, with 12 hr of light for 3 weeks. The animals were divided into 2 negative control groups (negative control 1, no yeast feedings; negative control 2, non-treated yeast feeding of 400 mg/kg), one positive control group (red-ginseng feeding of 400 mg/kg), and one test group. For the test group Geranti Bio-Ge Yeast®, (feedings of 100, 200, 400, 800 mg/kg) was administered with oral needles one time each day for 4 weeks. Ten mice from each of the 4 test groups were weighed and given 100-800 mg/kg (0.5-4 mL/kg) of freshly-prepared Geranti Bio-Ge Yeast®. Body weights were recorded prior to these test treatments and mortality was examined once a week.

Results and Discussion

The optimum cultivation condition to produce yeasts (Geranti Bio-Ge Yeast®) To determine the best nutrient conditions for culturing the yeasts, we created nutrient broths of different concentrations using peptone as a protein source, glucose and yeast extract as carbohydrate sources, as well as vitamins and minerals. We cultured the yeast *S. cerevisiae* in these different nutrient media at 30°C for 48 hr. The results showed that the culture medium containing 5.0% glucose, 0.3% peptone, and 0.3% yeast extract gave the greatest amounts of yeasts (Table 1).

The amount of organic-germanium produced based on the inorganic germanium amount added to the yeast culture As shown in Fig. 1, higher amounts of inorganic germanium added to the culture resulted in greater organic germanium accumulation in the yeasts. The rates of accumulation were 78.9, 75.2, 85.2, 92.2, 91.5%, in each test group in Table 3, respectively. The 4,000 ppm germanium concentration offered the best result with a 92.2% (3,690 ppm) accumulation rate. According to Song *et al.* (12), yeasts cultured in high concentrations of inorganic germanium produced more organic germanium. Since the experi-mental dosages of 1,000-5,000 ppm yielded a safe level of organic germanium based on the NOEAL (2,000 mg/kg in rats) (13), inorganic germanium concentrations of 3,000-5,000 ppm are considered to be adequate.

Table 1. The quantities of yeasts cultured in different nutrient broths

Glucose (%)	Peptone (%)	Yeast extract (%)	Amount of yeast (mg/mL)
1.0	0.1	0.1	41.6
2.0	0.1	0.1	44.5
3.0	0.1	0.1	49.8
4.0	0.1	0.1	41.4
5.0	0.1	0.1	52.2
5.0	0.2	0.1	50.9
5.0	0.3	0.1	56.7
5.0	0.4	0.1	53.3
5.0	0.5	0.1	53.6
3.0	0.3	0.2	53.5
3.0	0.3	0.3	67.4
3.0	0.3	0.4	58.8
3.0	0.3	0.5	53.3

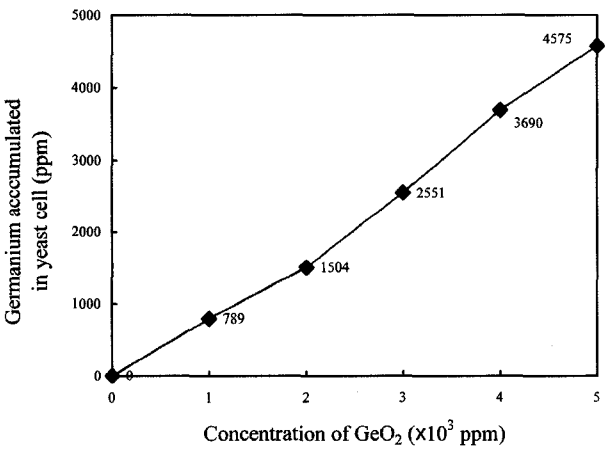

Fig. 1. Germanium accumulation in yeasts with changes in the amount of inorganic germanium added to the medium.

The effects of pH, temperature, and incubation time on the production of organic germanium in yeasts We studied the optimal conditions of pH, temperature, and incubation time for bio-germanium production in yeasts. Yeasts cultured in an optimal nutrient broth were mixed with inorganic germanium (1:1 v/v ratio) and then cultured at different pH levels, temperatures, and incubation times. Treatments of pH up to 6.5 increased the accumulation rate of germanium in yeasts, while a pH above 7.0 suppressed the production of bio-germanium. Temperatures up to 35 °C and incubation times to 20 hr increased the accumulation of bio-germanium, while temperatures and times above 40°C and 25 hr, respectively, suppressed the production. In summary, a pH of 6.5, a reaction temperature at 35°C, and 20 hr of incubation time were the best conditions to

produce maximum amounts of bio-germanium in yeasts (Table 2).

Changes in body weight with daily administrations of a control yeast, R-Gin, and various concentrations of Geranti Bio-Ge Yeast® Yeast is the safest and most effective organism to employ for producing organic germanium (14). To study the safety of organic germanium from the yeasts produced in this study, we performed acute oral toxicity tests in mice. Here we administered the bio-germanium yeasts orally once everyday. The body weights of the mice showed no significant differences within the test groups during the treatment period. Geranti Bio-Ge Yeast®, administered orally to mice for 4 weeks to concentrations of 800 mg/kg, did not induce any remarkable signs or affect body weight (Table 3 and Fig. 2).

Mortality of mice administered Geranti Bio-Ge Yeast® or control feedings While observing mortality during the feeding tests, only 3 mice died among a total of the 70 mice treated (including the control group) (Table 4); two mice were from the R-gin and Ger-100 treatment groups, respectively, and died the first week after administration. One mouse was from the Ger-200 treatment group and died at week zero. Mortality was found to be sporadic in the treatment groups, and it's possible this was due to a gavage error. As a result, no considerable differences among the treatment groups were obtained and it was concluded that Geranti Bio-Ge Yeast® had no effect on the mortality.

Organic germanium is safe and has excellent medical benefits, while inorganic germanium is toxic to the kidneys and liver (5). Therefore, the production of organic germanium is considered to be important. As so, we were motivated to

Table 2. The effects of pH, temperature, and time on the concentration of bio-germanium accumulated in the yeasts

pH	Temperature (°C)	Time (hr)	Germanium[1)] (ppm)
4.0	35	20	1,775
5.0	35	20	3,168
6.0	35	20	4,638
6.5	35	20	5,836
7.0	35	20	3,556
8.0	35	20	2,567
6.5	20	20	2,552
6.5	30	20	3,845
6.5	35	20	5,344
6.5	40	20	4,789
6.5	45	20	2,321
6.5	50	20	1,842
6.5	35	5	2,389
6.5	35	10	3,913
6.5	35	15	4,680
6.5	35	20	5,541
6.5	35	25	5,060
6.5	35	30	3,681

[1)]Concentration of bio germanium accumulated in yeasts.

Table 3. Body weight changes of 10 mice administered Geranti Bio-Ge Yeast® for 4 weeks

Group	Pre-administration			Post-administration				
	WK-3	WK-2	WK-1	WT 0	WT+1	WT+2	WT+3	WT+4
Control	14.53±0.98	14.18±2.46	18.81±1.04	21.01±0.58	21.99±0.50	22.59±0.95	22.78±0.87	21.86±1.29
Yeast	14.62±0.97	16.37±1.60	18.32±1.05	19.82±0.76	20.26±0.49	21.59±0.94	21.36±0.92	21.55±1.41
R-Gin	14.39±1.34	16.71±1.13	18.53±0.63	19.73±0.86	20.02±0.89	21.25±1.27	21.20±0.96	21.57±1.35
Ger-Y100	14.58±1.30	16.08±1.51	18.37±1.01	20.28±0.50	20.46±0.66	22.04±0.92	21.90±0.66	22.21±0.86
Ger-Y200	14.56±0.85	15.15±0.56	18.28±0.45	19.51±0.70	20.17±0.63	21.12±1.13	21.00±0.91	20.81±1.20
Ger-Y400	14.76±1.21	16.99±0.94	18.77±0.75	20.04±0.28	20.66±0.23	21.23±0.20	21.64±0.52	21.36±0.68
Ger-Y800	14.55±0.65	16.81±0.79	18.87±0.86	19.5±0.90	20.01±0.95	20.83±1.58	20.44±1.28	20.56±1.38

Table 4. Mortality of mice administered Geranti Bio-Ge Yeast® for 4 weeks

Group	Mouse No.	Pre-administration			Post-administration					Mortality % (Death/Total)
		WK-3	WK-2	WK-1	WK0	WK+1	WK+2	WK+3	WK+4	
Control	10	0	0	0	0	0	0	0	0	0
Yeast	10	0	0	0	0	0	0	0	0	0
R-Gin	10	0	0	0	0	1	0	0	0	10(1/10)
Ger-Y100	10	0	0	0	0	1	0	0	0	10(1/10)
Ger-Y200	10	0	0	0	1	0	0	0	0	10(1/10)
Ger-Y400	10	0	0	0	0	0	0	0	0	0
Ger-Y800	10	0	0	0	0	0	0	0	0	0

produce yeasts that contain high concentrations of bio-germanium by determining the optimal conditions for culturing yeasts and the best concentrations of inorganic germanium for the adaptation process. Generally, high concentrations of minerals limit the growth of micro-organisms, and additions of germanium into the culture media can decrease yeast growth and increase the difficulty of recovering inorganic germanium from the culture broth (15). By examining the culturing of yeast separately from the adaptation of yeasts to inorganic germanium, we were able to determine the optimal production conditions for producing yeasts with high concentrations of bio-germanium. In addition, this resulting method was very efficient for recovering inorganic germanium from the culture medium.

The germanium that accumulates in yeasts is mostly organic germanium (12, 14). It has been reported that bio-germanium chelates high molecular weight proteins and nucleic acids, preventing their passage through dialysis membranes. The bio-germanium yeasts produced in this study are expected to be useful for such diseases as cancer and rheumatic arthritis, as well as for increasing immunity in the human body. More detailed studies on the medical effects of bio-germanium yeasts are necessary in the future. In addition, its mechanisms of action should be studied in detail.

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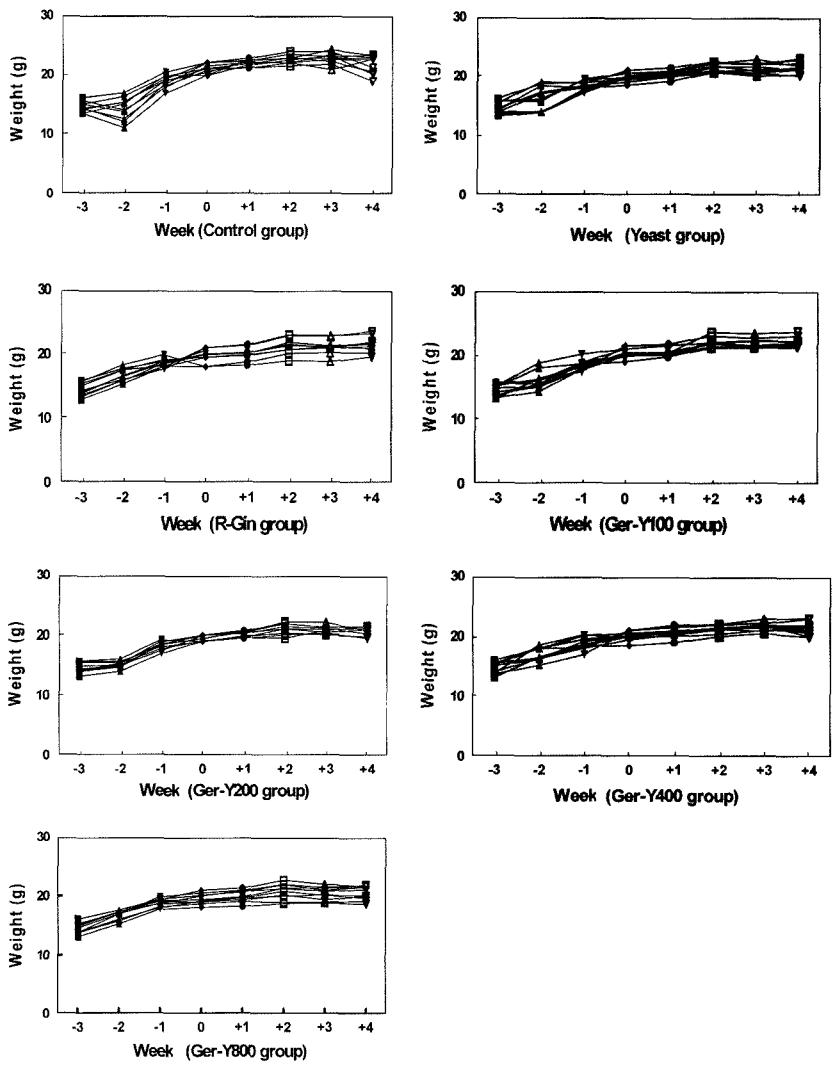

Fig. 2. Changes in the mean body weights of 10 mice during administration of red-ginseng and Geranti Bio-Ge Yeast®.

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