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Construction of a Magnesium-Enriched Yeast Strain and Study on Distribution of Magnesium in the Cells

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The fusant strains ZBF-23, ZBF-65, and ZBF-90 with both higher biomass and higher magnesium content in cells were constructed by protoplast fusion. These three fusant strains were used as the source of genome shuffling from which a magnesium-enriched yeast strain F3-3 with better ability to enrich magnesium was obtained. The enriched magnesium of the shuffled strain F3-3 was about 1.83 and 1.57 times of that of the primary strains ZB-29 and ZB-104, respectively. Under optimal fermentation condition, the biomass and magnesium content of cells reached 16.16 g L⁻¹ and 14.26 mg g⁻¹. In the total enriched magnesium which was incorporated into the yeast cells, the organic magnesium was about 95.7%, which was mainly in vacuoles and mitochondria. Nucleic acid and protein in the cells also combined 2.59 and 2.91 mg Mg²⁺ g⁻¹, respectively. The magnesium-enriched yeast strain which possesses higher ability to accumulate and convert inorganic magnesium salts could be used as a magnesium-fortified food supplement.

Key Words: biotransformation; distribution; genome-shuffling; magnesium content; *Saccharomyces cerevisiae*

INTRODUCTION

Magnesium is an essential mineral that participates in the natural physiological and metabolic process of organisms. It can affect various physiological functions of cells (He et al., 2006). Magnesium deficiency has been recognized to promote some disease states (Ames, 2006; Ferre et al., 2007). Epidemiological findings link a lowered magnesium status to many diseases, including cardiovascular diseases, hypertension, diabetes, osteoporosis, and

some cancers (Barbaqallo et al., 2007; Blache et al., 2006; Kanazawa et al., 2007; Larsson et al., 2005; Nielsen et al., 2007).

Magnesium is obtained mainly from drinking water and diet including green leafy vegetables, legumes, nuts, and unprocessed grains. The recommended dietary intake (RDI) for magnesium is 400–420 mg/day for adult men and 310–320 mg/day for adult women (Australian Government, 2006; Institute of Medicine, 1997). Intake estimates generated by some dietary surveys have indicated that many people in Western countries have magnesium intake below the RDI because the typical Western diet prefers more refined and often nutrient-poor food (Ames, 2006; Ford and Mokdad, 2003; McLennan and Podge, 1998; Saris et al., 2000). Moreover, the incidence and severity of magnesium inadequacy is even greater in special areas or at-risk groups, such as Australian Aboriginal and Torres Strait Islander people, the elderly, and people with alcoholism (Killilea and Ames, 2008). It has been found that low magnesium intake might be a potential contributor to diabetes in Australia, especially among Indigenous people (Longstreet et al., 2007). There is also a decline in magnesium intake among the elderly as total food intake, absorption, and utilization efficiency decline (Killilea and Maier, 2008; Wakimoto and Block, 2001). Owing to reduced dietary intake and enhanced renal excretion, significant magnesium deficiency occurs in the alcoholics (Brown, 1998). For these people, broadly correcting nutritional intake of magnesium is required to prevent the diseases relating to magnesium deficiency (Brown et al., 1998; Killilea and Ames, 2008).

It is well known that yeast has the enrichment ability for mineral elements. It can convert inorganic mineral to organic species. Organic species of mineral elements in cells are more suitable and bioavailable for both humans and animals than inorganic mineral (Blackwell et al., 1995; Hegoczki, 1994). Many mineral-enriched types of yeast have been studied. For example, the iron-enriched bakery' yeast had been obtained and the distribution of iron in the cells had been examined (Yuan et al., 2004). Demirci et al had enhanced the production of organically bound selenium and chromium yeast by continuous fermentation (Demirci and Pometto, 1999, 2000). Some mineral-enriched yeasts are available commercially, such as selenium-enriched yeast, chromium-enriched yeast, and so on. Yeast cells also can be used to enrich organic magnesium. Magnesium-enriched yeast has better absorption and less gastrointestinal side effect than magnesium gluconate which is used for oral magnesium supplementation (Swaminathan, 2003). Conway and Beary (1962) studied some chief properties of magnesium-enriched yeast. Wanda et al. (2005) investigated the capacity for natural binding of magnesium by baker's yeast. But the construction of high-biomass, magnesium-enriched yeast still has not been achieved.

The present investigation is mainly aimed at the construction of a magnesium-enriched yeast strain by protoplast fusion and genome shuffling

and analysis of the distribution of magnesium in the magnesium-enriched yeast cells.

MATERIALS AND METHODS

Strains

The 380 primary yeast strains from different genera and species were used in this study, designated as ZB-1 to ZB-380 strains (ZB-1 to ZB-200: *Saccharomyces cerevisiae*; ZB-201 to ZB-300: *Saccharomyces kluyveri*; ZB-301 to ZB-380: *Saccharomyces carlsbergensis*) and were obtained from Institute of Microbiology, Chinese Academy of Sciences. *Saccharomyces cerevisiae* A364(MAT α) and YF59(MAT α) were used as the standard mating-type strains.

Media and Culture Conditions

Seed culture was grown in YEPD (yeast extract, peptone, and dextrose) medium (Burke et al., 2000) for 16 h at 30°C with shaking at 200 rpm. For magnesium-enriched yeast production, the yeast strain was cultured in the optimized fermentation medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, 4% (w/v) glucose, and 0.7% (w/v) magnesium (magnesium acetate) for 35 h at 30°C with shaking at 200 rpm. Screening medium was YEPD solid medium supplemented with different concentration of magnesium. For sporulation, the strains were incubated in McClary slant medium (Burke et al., 2000) and incubated for 5–7 d at 30°C. Isolation of auxotrophic mutants was conducted on synthetic complete (SC) medium (Burke et al., 2000).

Chemicals and Reagents

Magnesium acetate was purchased from Beijing Chemical Plant (Beijing, China). DNase, trypsin, proteinase K, imidazole and MOPS were from Sigma Chemicals (St. Louis, Mo., USA). Helicase was from Biodee. All other chemicals were analytical grade.

Haploid Strains Preparation and Mutagenesis

Diploid yeast strains from the YEPD slants were transferred to sporulation medium slants and incubated for 5–7 d at 30°C. Sporulation was monitored by light microscopy. The sporulated cells were harvested by centrifugation and washed twice by sterile water. Then cells were suspended in sterile water and incubated at 58°C for 15 min to kill vegetative cells. Helicase was added to the suspension and incubated for 1 h at 37°C on a rotary shaker. Then the suspension was spread on YEPD plates and then incubated at 30°C. Single colonies were transferred to sporulation medium again and incubated for 5–7 d

at 30°C. Nonsporulated stains were considered to be haploid strains. They were transferred to YEPD plates and mixed with the standard mating-type strains A364 (*MATa*) and YF59 (*MAT α*), respectively. After 5 h at 30°C, the mating types (*MATa* or *MAT α*) of haploids were examined by light microscopy. Diethyl sulfate (DES) was used as the mutagenizing agent. Mutagenesis was performed on haploid strains. Auxotrophic mutants were obtained when the colonies could grow on YEPD medium but were unable to grow on SC medium.

Protoplast Preparation and Fusion

Auxotrophic mutants were cultured at 30°C for 16 h. Cells were harvested by centrifugation, washed twice with distilled water and suspended in 0.15 M phosphate buffer (pH 5.4) containing 0.01 M β -mercaptoethanol. After 30 min, cells were collected and then resuspended in 0.15 M phosphate buffer (pH 5.4) containing 1.0 M sorbitol. Helicase was then added to a final concentration of 10 mg/mL. After incubation for 60 min at 37°C, fresh protoplasts were washed with 0.15 M phosphate buffer (pH 5.4) containing 1.0 M sorbitol. Then protoplasts were mixed and resuspended in 0.15 M phosphate buffer (pH 5.4) containing 30% (w/v) polyethylene glycol (PEG; mol.wt4000) and 0.01 M CaCl_2 . After incubation for 40 min at 30°C, the suspension was slowly diluted with 0.15 M phosphate buffer containing 1.0 M sorbitol, and then embedded in SC medium containing 1.0 M sorbitol and incubated for 4 to 7 d at 30°C.

Genome Shuffling

The selected parental strains were mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) according to the method of Zhuge and Wang (1997). The appropriately diluted cells were plated on YEPD plates with 23.5 mg Mg^{2+} mL^{-1} and then incubated at 30°C. The mutants which could grow on YEPD plates with 23.5 mg Mg^{2+} mL^{-1} were picked. After analysis of the biomass and magnesium content of cells, the mutants that enriched more magnesium were obtained for protoplast fusion. Protoplast formation and fusion were carried out as described above. After incubation in YEPD medium containing 1.0 M sorbitol for 12 h at 30°C, the mixture was plated on YEPD plates with higher concentrations of magnesium and incubated for 2 d at 30°C. The fusants which could grow on YEPD plates with higher concentrations of magnesium were picked. After analysis of the biomass and magnesium content of cells, the fusants that enriched more magnesium were chosen for the subsequent round of genome shuffling. Subsequent rounds of genome shuffling were carried out by repeating the protoplast fusion described above.

Analysis of Genetic Stability of the Shuffled Strain

Yeast strain was successively transferred to YEPD slants for 10 generations. The culture of the 10th generation was diluted and spread on YEPD

plates. After incubation for 2 d at 30°C, 100 single colonies were chosen randomly and transferred to sterile water. After 4–6 h, each starved suspension was inoculated on YEPD plate containing 25 mg Mg^{2+} mL^{-1} and incubated for 2 d at 30°C. Sporulation, biomass, and magnesium content of cells of these colonies were determined.

Measurement of the Biomass and Magnesium Content of Yeast Cells

Cells were collected by centrifugation, washed three times with deionized water, and dried at 60°C to constant weight. Then the weight of cells was measured. The biomass was determined as the grams of cell dry weight per liter of culture (g L^{-1}). Cells were digested with 14 M nitric acid/10 M perchloric acid (4:1, v/v) by boiling the mixture for 40 min. The concentration of magnesium was determined by atomic absorption spectrometry. The magnesium content of cells was determined as the milligram magnesium per gram dry cells (mg g^{-1}). Each sample was analyzed three times.

Determination of Inorganic and Organic Magnesium Contents of Yeast Cells

Cells were washed twice with deionized water, suspended in 0.05 M phosphate buffer (pH 8.0), and sonicated (sonicate for 30 min total time. 10 sec “on,” 10 sec “off”). The suspension containing disrupted cells was then centrifuged at 6000 g for 8 min. The inorganic magnesium content of supernatant without digestion was determined by atomic absorption spectrometry. The magnesium content of cells minus the inorganic magnesium content of cells was the organic magnesium content of cells.

Separation of Yeast Cell Fractions

Cell wall fraction was prepared as described by Zhuge and Wang (1997). Vacuolar and mitochondrial fractions were obtained with the described method (Raguzzi et al., 1988). Cell membrane fraction was prepared according to the method of De Silva et al. (1995). Total protein of the cells was prepared with the method of De and Vanderleyden (1989) and determined using the routine Kjeldahl method. Nucleic acid was prepared according to the method of Burke et al (2000).

RESULTS

Strains Screening

A total of 28 yeast strains which could grow on YEPD plates containing 23 mg Mg^{2+} mL^{-1} were obtained from 380 yeast strains. The biomass

Table 1: Comparison of the biomass and magnesium content of primary yeast strains (partial data).

Strains	ZB-12	ZB-29	ZB-45	ZB-104	ZB-270	ZB-321
Biomass (g l ⁻¹)	12.32 ± 0.65	12.89 ± 0.48	9.63 ± 0.45	9.32 ± 0.67	9.05 ± 0.66	12.26 ± 0.55
Magnesium content of cells (mg g ⁻¹)	5.75 ± 0.81	5.62 ± 0.40	8.65 ± 0.69	9.19 ± 0.92	9.01 ± 0.55	5.21 ± 0.47

ZB-12, ZB-29, ZB-45 and ZB-104: *Saccharomyces cerevisiae*; ZB-270: *Saccharomyces kluyveri*; ZB-321: *Saccharomyces carlsbergensis*.

Values are means of three replicated experiments ± standard deviation.

and magnesium content of 28 strains were evaluated (Table 1). *Saccharomyces cerevisiae* ZB-29 with high biomass and *Saccharomyces cerevisiae* ZB-104 with high magnesium content of cells were used as parental strains in the following haploid preparation. Specifically, 12 and 15 haploid strains were obtained from *S. cerevisiae* ZB-29 and *S. cerevisiae* ZB-104, respectively. After analysis of biomass and magnesium content of cells, haploid *S. cerevisiae* ZB-29-33 with higher biomass and haploid *S. cerevisiae* ZB-104-51 with higher magnesium content were obtained. The mating-types of *S. cerevisiae* ZB-29-33 and *S. cerevisiae* ZB-104-51 were both determined as *MATa*.

Construction of High-Biomass, Magnesium-enriched Yeast Strains by Protoplast Fusion

S. cerevisiae ZB-29-33(*MATa*) and *S. cerevisiae* ZB-104-51(*MATa*) were both mutagenized by diethyl sulfate (DES). Auxotrophic mutants ZB-29-33-3(*MATa*, *his*) with high biomass and ZB-104-51-6(*MATa*, *met*) with high magnesium content were chosen as parental strains for protoplast fusion. Among 122 fusants (designated as ZBF-1 to ZBF-122), fusants ZBF-23, ZBF-65, and ZBF-90, which could tolerate magnesium ion less than 23 mg mL⁻¹ and had a higher ability for magnesium enrichment, were used as the source for genome shuffling (Table 2).

Improvement of Magnesium Tolerance and Magnesium Content of Yeast by Genome Shuffling

Yeast strains ZBF-23, ZBF-65, and ZBF-90 were mutagenized by NTG and plated on YEPD containing 23.5 mg Mg²⁺ mL⁻¹, respectively. A total of 5 single colonies which could grow on YEPD containing 23.5 mg Mg²⁺ mL⁻¹ and enriched more magnesium were selected and used as the starting population for the genome shuffling. The strains from each round of genome shuffling were named as F1, F2, and F3.

Table 2: Comparison of the biomass and magnesium content of cells among parental strains and the fusants.

Strains	Biomass (g l ⁻¹)	Magnesium content of cells (mg g ⁻¹)	Total enriched magnesium of per liter culture (mg l ⁻¹)
ZB-29	12.82 ± 0.50	5.60 ± 0.50	72
ZB-29-33	7.21 ± 0.85	4.02 ± 0.45	29
ZB-29-33-3	7.32 ± 0.35	4.57 ± 0.62	33
ZB-104	9.28 ± 0.42	9.08 ± 0.83	84
ZB-104-51	5.32 ± 0.80	6.82 ± 0.61	36
ZB-104-51-6	5.73 ± 0.32	6.63 ± 0.30	38
ZBF-23	11.12 ± 0.68	9.05 ± 0.72	101
ZBF-65	11.35 ± 0.48	8.95 ± 0.90	102
ZBF-90	11.44 ± 0.45	9.02 ± 0.57	103

ZB-29 and ZB-104: diploid strains; ZB-29-33 and ZB-104-51: haploid strains (*MATa*); ZB-29-33-3: auxotrophic haploid strain (*MATa*, *his*); ZB-104-51-6: auxotrophic haploid strain (*MATa*, *met*); ZBF-23, ZBF-65 and ZBF-90: fusant strains.

Strains were cultured in the initial fermentation medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 0.5% (w/v) magnesium (magnesium acetate)) for 40 h at 30°C with shaking at 200 rpm.

Values are means of three replicated experiments ± standard deviation.

After the first round of recursive protoplast fusion, seven strains (F1-1 to F1-7) were obtained that could grow on the plates containing 24.0 mg Mg²⁺ mL⁻¹ and enriched more magnesium than mutants that had been obtained in the mutagenesis with NTG. In the following successive rounds of protoplast fusion, the concentration of magnesium in YEPD plates for selection continued increasing to 24.5, 25.0 mg Mg²⁺ mL⁻¹. Five strains (F2-1~F2-5) and four strains (F3-1~F3-4) were obtained, respectively. Among the F3 shuffled strains, F3-3 was selected for its higher biomass and magnesium content of cells (Table 3). F3-3 exhibited 28–31% improvement in total enriched magnesium per liter culture medium than those of the parental strains ZBF-23, ZBF-65, and ZBF-90. The total enriched magnesium content of the strain F3-3 was about 1.83 and 1.57 times of that of the primary strains ZB-29 and ZB-104, respectively.

As a control experiment, the mutants, yeast strains F1 and F2 were plated on the YEPD plates containing 24.0, 24.5, and 25.0 mg Mg²⁺ mL⁻¹, respectively. After incubation, no colonies appeared which implied that there was no adaptive growth to lead to improved magnesium tolerance of yeast strains.

The genetic stability of the shuffled strain F3-3 was tested according to the method of He et al. (2000). After 10 generations growth, the single colonies of strain F3-3 did not produce spores on the sporulation medium and could grow on the YEPD solid medium containing 25mg Mg²⁺ mL⁻¹. The biomass and magnesium content of cells of all single colonies had no significant changes.

Table 3: Comparison of recombinant yeast strains for their abilities to enrich magnesium (partial data).

Strains	Biomass (g l ⁻¹)	Magnesium content of cells (mg g ⁻¹)	Total enriched magnesium of per liter culture (mg l ⁻¹)
ZB-29	12.82 ± 0.50	5.60 ± 0.50	72
ZB-104	9.28 ± 0.42	9.08 ± 0.83	84
ZBF-23	11.12 ± 0.68	9.05 ± 0.72	101
ZBF-65	11.35 ± 0.48	8.95 ± 0.90	102
F0-1	11.36 ± 0.48	9.51 ± 0.42	108
F0-4	11.15 ± 0.41	9.42 ± 0.51	105
F1-3	11.11 ± 0.49	10.09 ± 0.45	112
F1-5	11.12 ± 0.37	10.02 ± 0.45	111
F2-2	11.11 ± 0.42	10.72 ± 0.56	119
F2-3	11.08 ± 0.41	10.38 ± 0.37	115
F3-2	11.25 ± 0.52	11.20 ± 0.53	126
F3-3	11.62 ± 0.49	11.32 ± 0.73	132

ZB29 and ZB104: primary yeast strains; ZBF-23 and ZBF-65: starting strains in genome shuffling; F0-1 and F0-4: mutant strains; F1-3 and F1-5: strains from the first round of genome shuffling; F2-2 and F2-3: strains from the second round of genome shuffling. F3-2 and F3-3: strains from the third round of genome shuffling. Two strains were selected respectively as representative in each process in the table.

Strains were cultured in the initial fermentation medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 0.5% (w/v) magnesium (magnesium acetate)) for 40 h at 30°C with shaking at 200 rpm.

Values are means of three replicated cultures.

Optimization of Culture Conditions of Magnesium-Enriched *S. cerevisiae* F3-3

The influences of different culture conditions, such as different sugars and sugar concentrations, different magnesium salts and magnesium concentrations, volume of medium, and incubation time, on cell growth and magnesium enrichment ability of F3-3 were determined. The magnesium enrichment ability of strain F3-3 was greatly improved after the optimization of cultivation conditions. The highest magnesium yield was obtained when 20 mL of culture in 250-mL shake flask was grown in fermentation medium for 35 h at 30°C and 200 rpm. Under the optimal cultivation condition, the biomass and magnesium content of strain F3-3 reached 16.16 g L⁻¹ and 14.26 mg g⁻¹, respectively, which were 1.38 and 1.26 times when compared to those under the initial cultivation conditions. The proportion of organic magnesium was about 95.7%.

Distribution of Magnesium in the Magnesium-enriched *S. cerevisiae* F3-3

In the cells grown in the medium containing magnesium acetate, the magnesium content in organelles including cell membrane, vacuoles, mitochondria,

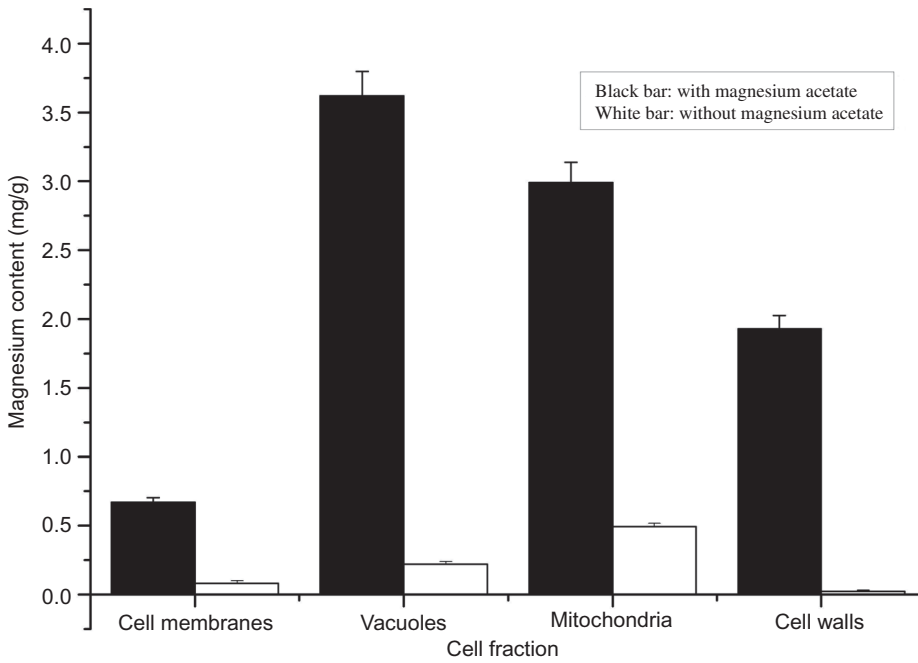


Figure 1: Distribution of organic magnesium in the magnesium-enriched yeast strain F3-3. Values are means of three replicated cultures. Bars represent standard deviation.

and cell wall was greatly higher than that in the cells grown in the medium without magnesium acetate. Particularly, the magnesium content in vacuoles and mitochondria accounted for 25% and 21% of the magnesium content in the cells, respectively (Fig. 1). It implied that the enriched magnesium mainly existed in vacuoles and mitochondria.

The magnesium content in nucleic acid and protein was determined when yeast cells were cultured in the medium with or without magnesium acetate, respectively. The results showed that the magnesium content of nucleic acid (2.59 mg g^{-1}) and protein (2.91 mg g^{-1}) in the former medium was much higher than those (nucleic acid: 0.308 mg g^{-1} ; protein: 0.588 mg g^{-1}) in the latter medium. In the organic magnesium in yeast cells, 17% and 19% were bound with nucleic acid and protein, respectively.

DISCUSSION

The production of magnesium-enriched yeast depends on not only magnesium content of cells, but also biomass. High biomass was accompanied by low magnesium content of cells (or high magnesium content of cells was accompanied by low biomass) in wild strains (Wanda et al., 2005). It was difficult to get a strain with both higher biomass and higher magnesium content of

cells by natural screening or mutagenesis. However, it was possible to obtain such strains by genetic breeding technology, such as protoplast fusion. In this study, ZB-29-33-3(*MATa*, *his*) with high biomass and ZB-104-51-6(*MATa*, *met*) with high magnesium content were chosen as parental strains for protoplast fusion. The fusants ZBF-23, ZBF-65, and ZBF-90 constructed by protoplast fusion had higher magnesium enrichment than their parental diploid strains ZB-29 and ZB-104. Then these three fusant strains were used as the source of the subsequent genome shuffling. The magnesium-enriched *S. cerevisiae* F3-3 with better ability to enrich magnesium was obtained by genome shuffling. Genome shuffling, using recursive multi-parental fusion and high-throughput screening, is an effective breeding method which permits the microbial strains to have desirable phenotype rapidly without knowing genome information of strains.

Under optimal conditions, the biomass and magnesium content of strain F3-3 was 16.16 g L⁻¹ and 14.26 mg g⁻¹. The organic magnesium was 95.7% of the total magnesium in the cell, which was in conformity with the result reported by of Heaton (1993). When absorbed by yeast cells, magnesium combines with many intracellular organelles including cell membrane, vacuoles, mitochondria and cell wall. Vacuoles display uptake and storage of some cations, which are physiologically useful but may be harmful at excess concentrations (Daniel et al., 1990). When cells incorporate excess magnesium, vacuoles may store numerous magnesium to maintain the normal metabolism of cells. ATP is a major chelating agent for magnesium (Heaton, 1993). As the main ATP producer, mitochondria may contain much magnesium. In this study, much magnesium existed in vacuoles and mitochondria of the cell. It implied that the enriched magnesium mainly existed in vacuoles and mitochondria. Meanwhile, there was considerable magnesium bound with nucleic acid and protein respectively. It suggested that magnesium bound preferentially with phosphorylated and nitrogenous structures (Heaton, 1993).

As organic magnesium, the magnesium absorption and bioavailability of magnesium-enriched yeast were better than those of inorganic magnesium. Hence, the magnesium-enriched yeast strain F3-3 could be used as a magnesium-fortified food supplement. For increasing the magnesium absorption rate and bioavailability of magnesium-enriched yeast, the further study on the combination pattern of magnesium with organelles of yeast cells and their biological activities is needed.

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