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Isolation and Identification of Zinc-Enriched Yeast *Saccharomyces cerevisiae* FF-10 from the Tropical Fruit Rambutan

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Zinc is an essential trace element in Human Being. Highly zinc containing yeast strain isolated from the tropical fruit, rambutan and the zinc concentration in this yeast strain was 306 ppm (30.6 mg%) per dry matter basis. This strain was found to be a rounded type, normal size, and multi-polar budding. Phylogenetic analysis using the ITS1-5.8S rDNA sequences from isolated strain is most similar to yeast *Saccharomyces cerevisiae* at the level of nucleotide sequence identity at 99%. This strain was produced alcohol by about 12% using fully colonized koji-rice with *Aspergillus oryzae*. In conclusion, the isolated strain was found to be closely related to the *S. cerevisiae* based on its morphological and physiological properties, and alcohol fermentation. The phylogenetic analysis of strain FF-10 using ITS 5.8S rDNA sequence data also supported the closely related to the *S. cerevisiae*. Accordingly, the isolated yeast was named as *S. cerevisiae* FF-10. Further studies on the best culture conditions for zinc production from zinc-enriched *S. cerevisiae* FF-10 are under investigation.

Key words : FF-10 strain, *Saccharomyces cerevisiae*, zinc, ITS1-5.8S rDNA sequences.

Introduction

Yeast has long been utilized in areas of food production such as brewing, wine making, and baking and in areas of medicine and cosmetic industry due to their many important bioactive components, such as amino acids (glycine, methionine etc.), minerals (zinc etc.), and polysaccharides (beta-glucan), peptides and other ingredients (glutathione, cerebroside) [15], terrein [19] and which have been found to protect hepatic injuries [9,21,28,29] as well as to reduce body weight and serum lipid levels in animals [22,30].

Many studies have tried to analyze microorganism effects, especially of yeast strains, in hepatic injury induced by hepatotoxicants, such as alcohol and carbon tetrachloride [26]. We also recently demonstrated that dietary supplementation of the glutathione-enriched *S. cerevisiae* FF-8 strain, which is isolated from traditional Korean rice wine, protected against carbon tetrachloride-induced hepatotoxicity and oxidative stress in rats [28].

Zinc is an essential trace element of all organisms including human [7], and also plays an important biological role in lipid metabolism, insulin resistance, immune response, antioxidant properties and hepatic injury [4,24,31], together with important function for sperm physiology [2,16]. Recent studies have shown that dietary zinc supplementation significantly increases plasma-free and total testosterone concentrations in rats and camels [1,4]. The recommended dairy intake (RDI) of zinc is 10 mg per day in Japan and 15 mg in the USA and the maximum intake not associated with adverse effect is 30 mg [13,26]. In the USA, 18% of people consume less than half the recommended levels of zinc [8]. The main cause of zinc deficiency in human is nutritional. Clinical zinc deficiency causes various clinical problem, such as growth retardation and taste impairment [20,26]. Zinc has shown hepatoprotective effects against hepatotoxins, chlorpyifos, and bromobenzene [5,11]. Therefore, we expected that a zinc-accumulating yeast strain would enhance suppressive effects against alcoholic liver injury, because zinc suppresses liver damage.

Rambutan (*Nephelium lappaceum* L.) is a native tropical fruit of Southeast Asia, and is a bright-red oval fruit with a sweet taste [6]. One yeast strain has been isolated from the juice of the rambutan fruit [23]; however, there is very limited information on the capacities of this high zinc-containing yeast strain on suppressing liver injury.

Thus, the current study tried to the isolation of the

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zinc-enriched yeast strain from the rambutan fruit expecting protective effect against hepatic injury.

Materials and Methods

Yeast strain isolation and culture conditions

The tropical fruit rambutan was purchased at a local market at Jan. 2006 in Bangkok, Thailand, at the fully mature stage. In other to isolating the zinc-enriched yeast strain, rambutan juice spread on YM agar plates (glucose 1.0% (w/v), peptone 0.3%, yeast extract 0.3%, malt extract 0.3%, and agar 1.5%), and incubated at 30°C for 72 hr. After incubation, each colony of yeast strains identified using an optical microscope was picked off and 500 ml flask containing 100 ml the same medium and then incubated at 30°C for 72 hr on a reciprocal shaker with the rotation speed of 200 rpm. The culture cells were centrifuged at 7,000× *g* for 15 min after the incubation and then the supernatant was removed and the yeast cells were washed three times with distilled water. Mineral concentrations in harvested yeast cells were analyzed using Shimadzu ASC-600 model Atomic Absorption Spectrophotometer. The highest zinc containing yeast strain among these was selected, and named FF-10. This FF-10 strain was maintained by monthly subcultivated on the YM agar medium plate.

Morphological characteristics

Morphological characteristics of the isolated FF-10 strain was examined by phase-contrast microscopy and Scanning electron microscopy (SEM). The cells were fixed with 2% glutaraldehyde (0.1 M cacodylate containing 0.1% $MgSO_4$ buffer, pH 7.2) and 1% Osmic acid (OsO_4) (0.2 M cacodylate buffer, pH 7.4). Ultrathin section of the sample embedded in epoxy resin were prepared with an ultramicrotome, stained with uranylacetate and lead citrate. SEM was taken using a model JSM35CF (JEOL Ltd., Tokyo, Japan).

PCR amplification of ITS1-5.8S rDNA

Fungal DNA spanning ITS1-5.8S rDNA was amplified with the two primers, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [33] using genomic DNA obtained from our strain FF-10 as the template. The PCR amplification was carried out under the following conditions: 94°C for 2 min initial denaturation; 25 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1 min, and final extension at 72°C for 5 min. The reaction mixture (final volume, 50 l) contained 1l DNA (approximately 4,050 ng), 20 mM Tris-HCl (pH 8.0), 40 mM NaCl, 2 mM sodium phosphate, 0.1 mM EDTA, 1 mM DTT, 0.2 mM each dNTP, 2 mM MgSO4, 0.25 M each oligonucleotide primer, and 2.5 U *Taq* DNA polymerase (New England BioLabs, Inc., Beverly, MA, USA). The amplified products corresponding to the correctly sized product was excised and ligated into the pGEM-Teasy vector (Promega, Madison, WI, USA) and transformed into *E. coli* DH5 competent cells. Subsequently, insert DNA were used for nucleotide sequence analysis. The nucleotide sequencing and DNA oligonucleotide synthesis were performed commercially at the DNA sequencing facility of GenoTech Corp. (Daejeon, Korea). The nucleotide sequences were compared using Blastn program provided by the National Center for Biotechnology Information (NCBI).

Phylogenetic analysis of sequence data

The identity of our strain was compared with other comparable sequences of Ascomycotina strains. The ITS1-5.8S rDNA sequences of 15 strains of Ascomycotina and our strain FF-10 were aligned with the CLUSTALW program [32] and visually examined with the GENEDOC program. Phylogenetic trees were constructed using DNADIST with the Jukes-Cantor model [10] and NEIGHBOR with the neighbor-joining method [25] in the PHYLIP (phylogeny inference package) programs, version 3.61. We generated 1,000 bootstrapped replicate resampling data sets with SEQBOOT (PHYLIP, version 3.61). We followed the standard protocol for the default settings of the computer programs used in this procedure.

Minerals analysis

Mineral concentrations in FF-10 strain were analyzed using ICP-AES (Inductively coupled plasma-atomic emission spectrophotometer) [34]. The measurements were repeated twice for each sample according to flame atomization technique. Mineral concentrations were presented as ppm/d.w. cells.

Compositional amino acid analysis

The compositional amino acids were determined with amino acid analyzer (Automatic Amino Acid Analyzer 3A30 Perkinelmer, USA) by using the acid hydrolysis and

ninhydrin procedure. The hydrolysis of freeze-dried yeast sample (10 mg) was placed into a sealed tube for 24 hr at 110°C, using 1 ml of 6 -N HCl containing 5 mg/ml of phenol. The hydrogenate was evaporated with a speed-vac concenter and the dried residue was dissolved in 0.5 ml of a citrate buffer. The samples were filtered with a 0.45 μm filter before being injected into the amino acid autoanalyzer.

Rice wine fermentation

The rice wine fermentation was prepared according to the methods described in previous papers[8,11,20]. Koji rice and FF-10 which was cultured in YMB medium at 30°C for 2 day were added and then the mixture fermented at 25°C for 12 day. After fermentation, the mixture was centrifuged to obtain the rice wine. Alcohol concentration was determined using an alcoholic meter, densitometer (Ceti Optical Instruments, Belgium) after water distillation [27].

Result and Discussion

Isolation of highly zinc producing yeast strain

The highly zinc producing yeast strain was isolated from the juice of tropical fruit rambutan and was named FF-10. The zinc concentration in FF-10 strain was 30.6 mg% (306 ppm) per dry weight cells (Table 1). Other studies were observed that the zinc concentration was active dry yeast by 5.2~6.7 mg% [34], beer brewing dried yeast by 4.0 mg% [30] and yeast *S. cerevisiae* strain by 0.55 mg% [2], and waste brewery yeast was identified as a trace element [3]. Zinc concentration in this study was comparatively higher than other results obtained previously [2,3,30,34]. The highest mineral composition was potassium by 2,864 mg%, followed by magnesium, sodium, calcium, and iron (Table 1).

Table 1. Concentrations of minerals in yeast FF-10 strain

Minerals (ppm)	FF-10	FF-8[1)]
Iron (Fe)	236.0	107.6
Copper (Cu)	4.0	2.0
Zinc (Zn)	306.0	189.7
Calcium (Ca)	366.0	39.2
Manganese (Mn)	4.0	2.8
Magnesium (Mg)	2,342.0	1,013.3
Sodium (Na)	1,048.0	94.1
Potassium (K)	28,640.0	22,390.0

[1)]FF-8 is yeast strain, glutathione-enriched *S. cerevisiae* established in our laboratory (22).

Morphological characteristics of yeast FF-10 strain

Yeast FF-10 strain was found to be a rounded type, normal size of 4.5~3.4 μm [23], multi-polar budding, as shown in Fig. 1.

Identification of yeast FF-10 strain

The almost complete DNA sequence analysis of whole PCR product spanning ITS1-5.8S rDNA-ITS2 revealed that our strain FF-10 is highly similar to *S. cerevisiae* at the level of nucleotide sequence identity to 99% (Fig. 2). The ITS regions and the 5.8S rDNA region are useful in measuring the phylogenetic relatedness and also in identification of fungal species because they exhibit great interspecies variations. Phylogenetic analysis using the ITS1-5.8S rDNA sequences from 15 strains of Ascomycotina including a strain of *S. cerevisiae* indicated that our strain FF-10 is most similar to yeast *S. cerevisiae* (Fig. 3). Therefore, we identified our yeast strain as a *S. cerevisiae*.

Alcohol fermentation characteristics

Yeast *S. cerevisiae* has long been utilized in areas of food production such as brewing and wine making [17]. The molecular mass of ADH (Alcohol dehydrogenase) from FF-10 strain was identified have about 40 kDa by Western blot analysis, suggesting this ADH molecular mass showed very slightly larger than the ADH molecular mass of 40 kDa of the purified *S. cerevisiae* (data not shown). Alcohol concentration was maximally produced to 11.63% by the fermentation using fully colonized koji-rice with *Aspergillus oryzae* for 12 day at 25°C. This alcohol productivity was lower than that of rice wine fermented by *Saccharomyces cerevisiae* isolated traditional rice wine which was produced to 16.07% under the same condition. However, it was

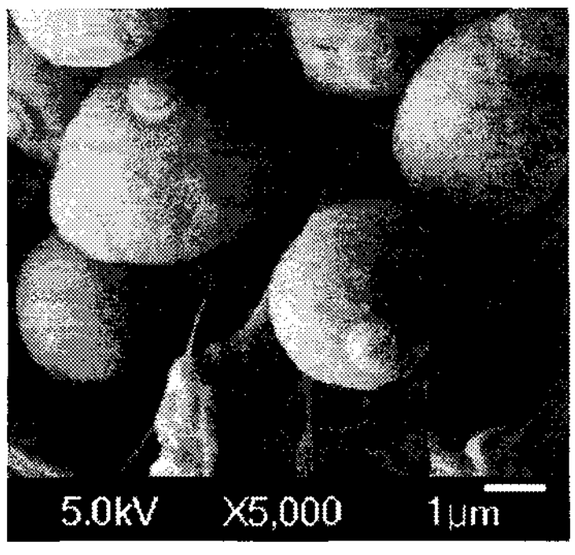

Fig. 1. Scanning electron micrograph of isolated highly zinc containing yeast FF-10 (×5,000). Scale bar: 1 μm.

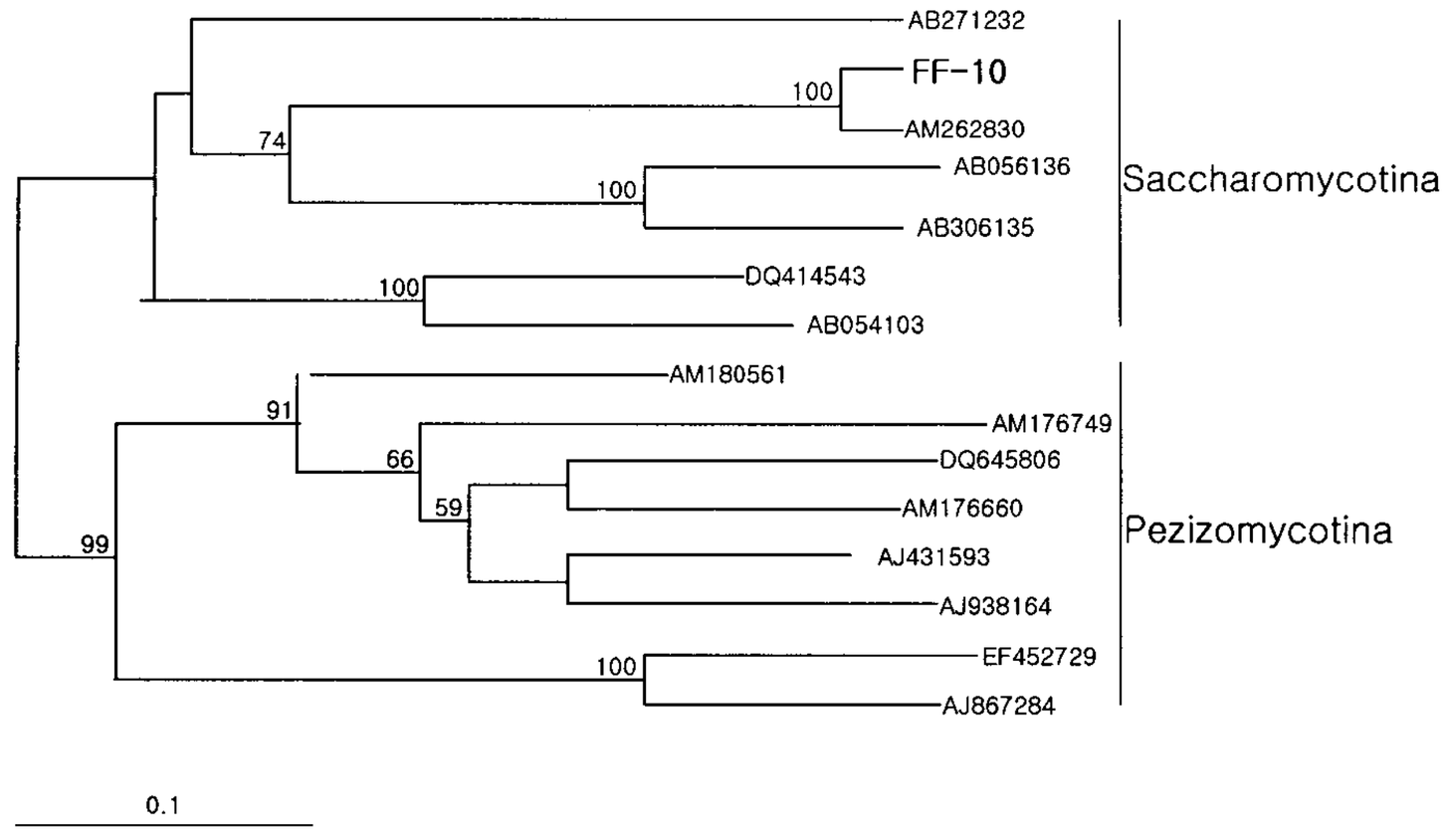

Fig. 2. Phylogenetic tree by ITS1-5.8S rDNA analysis of Ascomycotina strains and *Saccharomyces cerevisiae* FF-10. The GenBank accession numbers for the used sequences were included in parenthesis. Bootstrap values are shown for each node that had > 50% support in a bootstrap analysis of 1,000 replicates. The scale bar indicates 0.1 change per nucleotide substitutions per site. Accession numbers of the species shown in this tree are: *Saccharomyces cerevisiae* CBS405 (AM262830), *Hanseniaspora occidentali* IFO 1819 (AB056136), *Saccharomycodes ludwigii* ATCC 44299 (AB056135), *Eremothecium coryli* (AB271232), *Pichia verona* (DQ414543), *Debaryomyces polymorphus* var. africanus (AB054103), *Penicillium* sp. NRRL 35648 (DQ645806), *Geomyces luteus* (AJ938164), *Phaeosphaeria avenaria* f. sp. triticae (EF452729), *Alternaria tenuissima* (AJ867284), and *Aspergillus sydowii* (AM176660).

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FF-10          -AGGACCATTAAAGAAATTTAATAATTTTGAAAATGGATTTTTTTGTTTTGGCAAGAGCA 59
AM262830       AAGGATCATTAAAGAAATTTAATAATTTTGAAAATGGGTTTTTTTGTTTTGGCAAGAGCA 60
                ****  ********************************* ***********************

FF-10          TGAGAGCTTTTACTGGGCAAGAAGACAAGAGATGGAGAGTCCAGCCGGGCCTGCGCTTAA 119
AM262830       TGAGAGCTTTTACTGGGCAAGAAGACAAGAGATGGAGAGTCCAGCCGGGCCTGCGCTTAA 120
               ************************************************************

FF-10          GTGCGCGGTCTTGCTAGGCTTGTAAGTTTCTTTCTTGCTATTCCAAACGGTGAGAGATTT 179
AM262830       GTGCGCGGTCTTGCTAGGCTTGTAAGTTTCTTTCTTGCTATTCCAAACGGTGAGAGATTT 180
               ************************************************************

FF-10          CTGTGCTTTTGTTATAGGACAATTAAAACCGTTTCAATACAGCACACTGTGGAGTTTTCA 239
AM262830       CTGTGCTTTTGTTATAGGACAATTAAAACCGTTTCAATACAACACACTGTGGAGTTTTCA 240
               ******************************************* ******************

FF-10          TATCTTTGCAACTTTTTCTTTGGGCATTCGAGCAATCGGGGCCCAGAGGTAACAAACACA 299
AM262830       TATCTTTGCAACTTTTTCTTTGGGCATTCGAGCAATCGGGGCCCAGAGGTAACAAACACA 300
               ************************************************************

FF-10          AACAATTTTATTTATTCATTAAATTTTTGTCAAAAACAAGAATTTTCGTAACTGGAAATT 359
AM262830       AACAATTTTATTTATTCATTAAATTTTTGTCAAAAACAAGAATTTTCGTAACTGGAAATT 360
               ************************************************************

FF-10          TTAAAATATTAAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCA 419
AM262830       TTAAAATATTAAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCA 420
               ************************************************************

FF-10          GCGAAATGCGATACGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGC 479
AM262830       GCGAAATGCGATACGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGC 480
               ************************************************************

FF-10          ACATTGCGCCCCTTGGTATTCCAGGGGGCATGCCTGTTTGAGCGTCATTTC 530
AM262830       ACATTGCGCCCCTTGGTATTCCAGGGGGCATGCCTGTTTGAGCGTCATTT- 530
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Fig. 3. The sequence of 5.8S rDNA region. Sequence identity is indicated by asterisk (*).

slightly lower to that of yakju by 12.6% using koji rice by fermentation period of 15 day at 25°C [14]. Accordingly, these morphological and physiological characteristics suggested that the yeast FF-10 strain was closely similarity *S. cerevisiae*.

Concentrations of compositional amino acid

Amino acids such as glutamic acid, glycine and alanine have hepatic protective effects against hepatotoxicity [18]. Recent study on application of silk fibroin, which is mostly composed of glycine by 42% and alanine by 32%, showed that it has liver protective effect against alcohol-induced hepatotoxicity [12]. Thus, present study was also analyzed the compositions of constituted amino acids from FF-10 strain. In the analyzed results of constituted amino acids for FF-10 strain, major compositional amino acids (% per 100 g dry weight) were glutamic acid by 8.24, aspartic acid by 3.39, lysine 3.37, and leucine by 3.30 (Table 2). In our previous study, a major compositional amino acid in *S. cerevisiae* FF-8 strain was also glutamic acid by 11.51 (% per 100 g dry metter basis) [28].

In conclusion, the strain FF-10 was found to be closely related to the *S. cerevisiae* based on its morphology, physiological properties, and alcohol fermentation. The phylogenetic analysis of strain FF-10 using ITS16S rDNA sequence data also supported the isolate closely related to the *S. cerevisiae*. Accordingly, the isolated yeast FF-10 was named as *S. cerevisiae* FF-10. Thus, the zinc-rich ingredient using zinc-enriched *S. cerevisiae* FF-10 isolated from the tropical fruit rambutan has great potential as functional food products for the hepatic injury agents. Further studies on the best culture conditions of various carbon, nitrogen, minerals, and vitamins sources against cell growth and zinc production from zinc-enriched *S. cerevisiae* FF-10 are under investigation.

Table 2. Concentrations of compositional amino acid in yeast FF-10

Amino acids (%)	FF-10	FF-8[1)]
Aspartic acid	3.39	5.46
Threonine	2.43	2.32
Serine	2.53	2.46
Glutamic acid	8.24	11.52
Proline	1.96	1.86
Glycine	2.85	2.93
Alanine	2.59	3.59
Cystine	-[2)]	-[2)]
Valine	3.23	5.95
Methionine	-[2)]	-[2)]
Isoleucine	2.34	3.02
Leucine	3.30	4.09
Tyrosine	1.61	1.92
Phenylalanine	2.25	2.42
Histidine	2.10	1.51
Lysine	3.37	4.58
Arginine	3.29	3.29

[1)]FF-8 is yeast strain, glutathione-enriched *S. cerevisiae* established in our laboratory (22).
[2)]Not detected.

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초록 : 열대과일 Rambutan으로부터 아연 고함유 효모 *Saccharomyces cerevisiae* FF-10 분리 및 특성

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아연은 사람에 있어서 필수미량 원소 중의 하나이다. 열대과일 Rambutan으로부터 아연 고함유 효모를 분리하였으며, 이 균주의 아연 함량은 306 ppm (30.6 mg%)이었다. 아연 고함유 효모는 전형적인 둥근 모양과 보통 크기로 다출아 형태를 취하고 있었다. 분리 균주의 동정을 위하여 ITS1-5.8S rDNA sequences를 실시한 결과 효모 *S. cerevisiae* 와 99%의 높은 상동성을 보였다. 또한 *Aspergillus oryzae*로 코지를 만들어 알코올 발효를 시킨 결과 12% 정도의 알코올 수득을 얻을 수 있었다. 이상의 결과에서 신규 분리된 효모는 형태학적 및 생리학적 특성과 알코올 발효 특성을 가지는 것으로 보아 *S. cerevisiae*와 거의 일치하여 *S. cerevisiae* FF-10으로 명명하였다. 향후 실험에서는 *S. cerevisiae* FF-10 균주가 세포내에 아연을 최대로 축적할 수 있는 배양 실험조건을 확립할 필요성이 제기된다.