The Antioxidative Activity of Glutathione-Enriched Extract from *Saccharomyces cerevisiae* FF-8 in *In Vitro* Model System

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The Antioxidative activities of the cell free extracts containing high glutathione by *Saccharomyces cerevisiae* FF-8 were tested *in vitro* experimental models : DPPH method for radical scavenging activity, ferric TBA method and ferric thiocyanate method using linoleic acid and tissue microsome for lipid peroxidation inhibitions. The concentration of intercellular glutathione by cultivating *S. cerevisiae* FF-8 in the YM optimal medium obtained 204 μg/mL, which was increased by 2.76-fold from 74 μg/mL in the YM basal medium. A comparition between the YM basal medium and the YM optimal medium on antioxidative substance produced by *S. cerevisiae* FF-8 was investigated. In DPPH (α,α'-diphenyl-β-picrylhydrazyl) method, the electron donating activity of the glutathione produced by *S. cerevisiae* FF-8 cultured in the YM optimal medium was as high as that of BHT (0.05%, w/v). The antioxidative activity was measured by inhibition against lipid peroxidation of rat tissues' microsomes. The results of antioxidant activity of the cell free extracts by *S. cerevisiae* FF-8 cultured in the YM optimal medium was shown in the following order : liver 60.98% > kidney 56.43% > heart 52.91% > brain 52.13% > testis 45.57% > spleen 42.95%. In antioxidative activities determined by ferric thiocyanate method and TBA methods against lipid peroxidation, the lipid peroxidation in the control mixture increased more rapidly than the typical peroxidation curve of linoleic acid from one day. The antioxidative activity of the cell free extracts by cultivating *S. cerevisiae* FF-8 in the YM optimal medium were higher than that of the YM basal medium. These data indicate that the cell free extracts containing a high intercellular glutathione of *S. cerevisiae* FF-8 cultured in YM optimal medium showed strong antioxidative capacities by DPPH radical scavenging activity and ferric thiocyanate and TBARS measurements.

Key words – *Saccharomyces cerevisiae* FF-8, Glutathione, Antioxidation, DPPH (α,α'-diphenyl-β-picrylhydrazyl), TBARS

Glutathione of reduced form is a well known thiol-containing tripeptide in animals, plants and microorganisms. Intracellular glutathione widely affects biological oxidation and reduction reactions in organisms[20]. Glutathione is an important component as intercellular protect against radiation[28], heavy metals[24] and many exogenous organic substances[12]. It also plays a sacrificial defense role against oxidative damage in organisms[3]. As glutathione is now widely used in medicine and functional health food for liver injury, and even cosmetic industry, the commercial demand for glutathione has been expending.

Free radicals play a major role in the pathogenesis of various diseases including atherosclerosis, inflammation, carcinogenesis, diabetes, brain dysfunction and hepatic injury [14]. Free radical scavengers are expected to be useful as preventive and therapeutic agents for various diseases. Recently, a few number of research was done on the existence of the antioxidative substance in microorganisms [15]. Many kinds of natural antioxidants, such as pyridoxantin, bisorbicillinol[1] and benzastatins[18] have been isolated from microorganisms. The antioxidative substances were also produced intercellularly into *S. cerevisiae* IFO 2114 and extracellularly into the culture medium by *Aspergillus soja* and *Aspergillus oryzae*[25]. Strong inhibitory effect on peroxide formation with fish oil reaction was also noted by the aforesaid microorganisms[17]. These observations indicated that the microorganisms possibly produced antioxidative substance to the environment. On the basis of this assumption, *Bacillus* sp. FF-7 was cultivated in optimal medium consisting of 2% galactose and 1% tryptone and an antioxidative activity was found in the culture fluid[8].

Many microorganisms have evolved a series of antioxidant mechanisms to maintain and protect their inter-

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cellular redox homeostasis[16]. These defences include the synthesis of antioxidants such as glutathione, ubiquinol, ascorbic acid, and tocopherols[13,29]. Recently several studies have described glutathione producing yeast strains, *Saccharomyces cerevisiae*, which is commonly used for commercial glutathione production with the expanding commercial demand for glutathione[26,30]. Our previous study isolated a high-glutathione producing yeast strain. It was identified as *Saccharomyces cerevisiae* based on the morphological, physiological and biochemical characteristics, and was designated as FF-8 from Korean traditional rice wine. The glutathione concentration produced by this strain was 74 μg/mL in the YM medium at 30℃ and 100 rpm for 72 hr[23]. We have also found that the productivity of glutathione as antioxidative substance by *S. cerevisiae* FF-8 in the YM basal medium increased by 2.76-fold by cultivating the same strain in the YM optimal medium consisting of 3.0% glucose as carbon source, 3.0% yeast extract as nitrogen source, 0.06% KH_2PO_4 as salt source and 0.06% L-cysteine as precursor amino acid of glutathione[9]. Several analytical methods have been proposed for the determination of total antioxidant capacity of biological materials. The generally preferred methods of determiming the antioxidant activity are the measurements of radical scavenging activity using discoloration of DPPH and lipid peroxidations inhibition by TBA method and thiocyanate method using linoleic acid[6]. The radical scavenging activity measurement using discoloration of DPPH has been widely used due to its stability, simplicity, and reproducibility[2]. Lipid peroxidation is one of the most common oxidation reactions and is involved in a diversity of biological phenomena[27]. Catalyzed Fe^{2+} and ascorbate to inhibit lipid peroxidation. TBA methods using has been also widely used, because food spoilage and *in vivo* membrane damage attributed to lipid peroxidation[2].

In this study, therefore, the antioxidative activity of the glutathione-enriched cell free extracts from *S. cerevisiae* FF-8 was tested *in vitro* experimental models, DPPH-scavenging receiving activity, ferric thiocyanate and TBARS methods against lipid peroxidation using rat tissues microsomal fractions and linoleic acid.

Materials and Methods

Materials

Thiobarbituric acid and DPPH were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents were of the best commercial grade available.

Cell culture

Saccharomyces cerevisiae FF-8, a glutathione producing yeast strain, was aerobically cultivated either in the YM optimal medium[9] consisting of 3.0% glucose as carbon source, 3.0% yeast extract as nitrogen source, 0.06% KH_2PO_4 as salt source and 0.06% L-cysteine as precursor amino acid of glutathione or the YM basal medium[23] consisting of 1.0% glucose, 0.5% peptone, 0.3% yeast extract and 0.3% malt extract at 30℃ and at 100 rpm for 72 hr. After incubation, the culture was centrifuged at 7,000 x g for 15 min, the supernatant removed, and the yeast cells washed with distilled water three times. The harvested yeast cells were suspended in 0.2 M phosphate buffer (pH 7.2) and disrupted by sonication. The disrupted cells were then removed by centrifugation, and the cell-free extracts containing a high glutathione by *S. cerevisiae* FF-8 obtained for *in vitro* antioxidative activity measurement.

DPPH radical scavenging activity assay

Antioxidative activity was determined based on the radical scavenging activity of the experimental compounds[5,6]. DPPH (1,1-diphenyl-2-picryhydrazyl) by 16 mg was disolved in ethanol by 100 mL and this solution was filtered with Whatman filter paper No. 2. After mixing 1 mL of DPPH solution with 5 mL of the cell-free extracts containing glutathione of *S. cerevisiae* FF-8, for 30 min incubation period at room temperature, the optimal density (OD) was measured at 528 nm. Results were expresed as a percentage DPPH-radical scavenging activity of the cell-free extracts of *S. cerevisiae* FF-8 cultured in both the YM basal and optimal mediums and were calculated according to the following equation : [DPPH radical scavenging activity (%) = (control OD-sample OD)/control OD×100]. BHT was used as positive control.

Lipid peroxidation at linoleic acid

The measurement of antioxidative activity of the cell-free extracts of *S. cerevisiae* FF-8 was performed by the ferric thiobarbituric acid (TBA) method, based on the monitoring of inhibition of linoleic acid peroxidation. The TBA method was used for lipid peroxidation of the linoleic acid using Fe^{2+}-ascorbate system as the oxidative catalysis[10,11]. The cell-free extracts of *S. cerevisiae* FF-8 was added to a solution of linoleic acid 0.13 mL, ethanol 10 mL, 0.01% $FeSO_4$

0.2 mL, 0.01% ascorbate 0.2 mL, and 0.2 M phosphate buffer (pH 7.0) 10 mL. The total reaction volume was adjusted to 20 mL with deionized distilled water, and the reaction mixture was incubated at 40℃ in the dark during up to 7 days. Every day, 0.2 mL of incubated reaction solution was mixed with 0.05 mL of 7.2% butylated hydroxytoluene and 2 mL of 20 mM thiobarbituric acid/15% trichloroacetic acid. The sample was mixed using a vortex, and incubated in a boiling water bath for 30 min to develop color. After cooling in cold water, the samples were centrifuged at 3,000 x g for 15 min, the absorbance of the resulting upper layer was measured at 500 nm. Deionized distilling water substituted for FF-8 cell-free extracts in the blank samples.

Preparation of tissues homogenate and subcellular fractions.

The eight week-old male Sprague-Dawley rats were allowed free access to a commercially available diet and water for 1 week and killed by withdrawing blood from the abdominal aorta, under light diethyl ether anesthesia. Their tissues were quickly removed and eventually used to estimate lipid peroxidation. The preparation of tissues from individual rat was homogenized in ice-cold 0.25 M sucrose solution containing 10 mM Tris-HCl buffer (pH 7.4), 1 mM ethylenediamine tetraacetate (EDTA) in IKA-ULTRA-TURRAX T25 basic homogenizer (IKA-WERKE GMBH & CO. KG, Staufen, Germany). The microsomal fractions were prepared as described previously[26], and were finally suspended in homogenizer buffer, at a concentration of approximately 1 mg of protein in 0.1 mL suspention. Protein concentrations were measured by the method of Lowry *et al.* using bovine serum albumin as a standard[19]. The microsomal fractions were used to analysis the concentrations of thiobarbituric acid reacting substances (TBARS).

Determination of lipid peroxidation (TBARS)

The lipid peroxidation in various tissues microsomal fractions induced with 0.2 mM Fe^{2+} and 0.25 mM ascorbate measured as concentrations of TBARS by the previously described method with a minor modification[21]. Tissues microsomal fractions containing Fe^{2+} and ascorbate with or without the cell free extracts, were placed in a shaking water bath at 37℃ for 30 min. And then equal volumes of 0.75% TBA and 15% trichloroacetic acid were added. Reaction mixtures were heated at boiling water for 30 min, after cooling in ice-water, centrifuged for 10 min at 3,000 rpm to separate corpuscolate particles. The absorbance of the supernatant was read at 532 nm. The concentrations of TBARS expressed as nanomoles of malondialdehyde (MDA) as a good biomarker of oxidative stress. BHT by 0.05% was used as positive control and which is one of the most wieldy used chemicals to retard oxidation.

Results and Discussion

The cell free extracts of glutathione-enriched *S. cerevisiae* FF-8 cultured in both the YM basal medium and the YM optimal medium were evaluated for antioxidant activity by *in vitro* experimental methods. The antioxidative activities were measured by DPPH-radical savenging activity and by inhibitions against lipid peroxidation of rat tissues microsomal fractions and lipid peroxidation using linolenic acid. The glutathione concentration achieved in YM optimal medium increased 2.76-fold by 204 μg/mL compared to YM basal medium by 74 μg/mL.

DPPH-radical savenging activity

It is well known that DPPH has been used as a generator of peroxy radicals *in vitro* system due to its stability, simplicity and reproducibility. Generally, natural antioxidants, such as glutathione, vitamin E, phenolic compounds, demonstrated great antioxidant action in DPPH radical scavenging activity [6,31]. A significantly positive relationship between phenolic compounds and DPPH radical scavenging activity was observed and this was derived from the effecitive hydrogen donors in phenolic compounds[31]. In this regard, the glutathione of *S. cerevisiae* FF-8 was subjected to DPPH radical scavenging assay. The radical scavenging activity was determined from the reduction in the optical absorbance at 523 nm due to the scavenging of stable DPPH free radicals.

The DPPH-radical savenging activity of the cell free extracts of glutathione-enriched *S. cerevisiae* FF-8 in the optimal medium by 67.26% was higher by 10% compared with that of the YM basal medium by 55.49% (Fig. 1). However, BHT (butylated hydroxytoluene, 0.05%) used as standard, showed a high antioxidant activity. The glutathione concentration produced by *S. cerevisiae* FF-8 wine was significantly increased by 2.76-fold by 204 μg/mL in the YM optimal medium compared to that of YM basal medium by 74 μg/mL. Thus, the antioxidant activity of *Saccharomyces cerevisiae* FF-8 showed to be in a proportion to the amount of the glutathione of the cell free extracts from cultured yeast strains

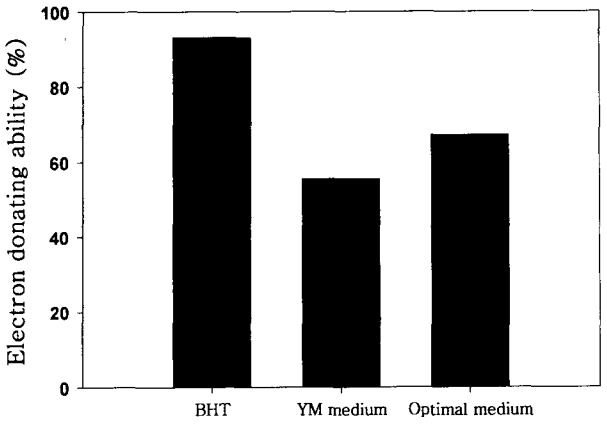

Fig. 1. DPPH radical scavenging activity of the cell-free extracts containing a high glutathione *Saccharomyces cerevisiae* FF-8 in both the YM basal and the optimal mediums. BHT : butylated hydroxytoluene (0.05%).

in both the YM optimal and YM basal mediums. The potent antioxidant activity of a high-glutathione producing strain, *S. cerevisiae* FF-8, may be derived from the intercellular glutathione amount and may act as primary antioxidants that react with peroxy radicals.

Inhibition of lipid peroxidation in tissues microsomes

Glutathione is important in cellular mechanisms of protection against lipid peroxidation. Glutathione also plays a sacrificial defense role against oxidative damage in organisms[3]. The inhibition activity of Fe^{2+}-catalyzed lipid peroxidation by the ferric TBA method has been widely used due to *in vivo* membrane damage attributed to lipid oxidation[2]. The antioxidative activities of the intercellular glutathione containing cell free extracts from *S. cerevisiae* FF-8 cultured in both the YM basal medium and the optimal medium on Fe^{2+}-catalyzed lipid peroxidation inhibition by TBA method were expressed as the percentage degreese of TBARS related to control. The relationship between the intercellular glutathione concentrations of *Saccharomyces cerevisiae* FF-8 in both the YM basal medium and the optimal medium and DPPH radical-scavenging activity was observed to have a significant positive correlation.

Metal ions such as Fe^{2+} and Cu^{2+} have been known to catalyze the lipid peroxidation. Virtually, all cellular components such as lipids, proteins, carbohydrates and nucleic acids are known to undergo metal-catalyzed oxidative modification[22]. The lipid peroxidation process is initiated by the abstraction of a hydrogen atom in an unsaturated fatty acid chain and propagated as a chain reaction during the lipid peroxidation of membranes[14]. Therefore, the inhibition of lipid peroxidation in the initial stage is of great importance in the protection of disease processes involving free radicals. Hence, present study examined the inhibitory effect of glutathione-enriched *S. cerevisiae* FF-8 on lipid peroxidation of various tissues (liver, heart, kidney, spleen, brain and testis), and microsomal fractions caused by Fe^{2+}-catalyzed system. The antioxidative activity of the glutathione produced by *S. cerevisiae* FF-8 in the YM optimal medium was shown in the following order : liver 60.98%, kidney 56.43%, heart 52.91%, brain 52.13%, testis 45.57% and spleen 42.95% (Fig. 2). As expected, BHT treatment used as standard control showed a high antioxidative activity. Interestingly, the lipid peroxidations of all tissues microsomal fractions were strongly suppressed in the cell free extracts containing intercellular glutathione by cultivating *S. cerevisiae* FF-8 in the YM optimal medium as comparition with that in the YM basal medium. The significant positive relationship between glutathione concentrations and lipid peroxidation inhibition was also observed. The glutathione concentration of *S. cerevisiae* FF-8 in the YM optimal medium markedly increased by 2.27-fold compared with that in the YM basal medium. These indicate that glutathione is strong antioxidative substance and functions as effectively as natural antioxidant in *S. cerevisiae* FF-8.

Inhibition of lipid peroxidation at linoleic acid

Lipid peroxidation is one of the most common oxidation reactions and is involved in a diversity of biological phenomena[27]. In the search for antioxidants, the inhibition of

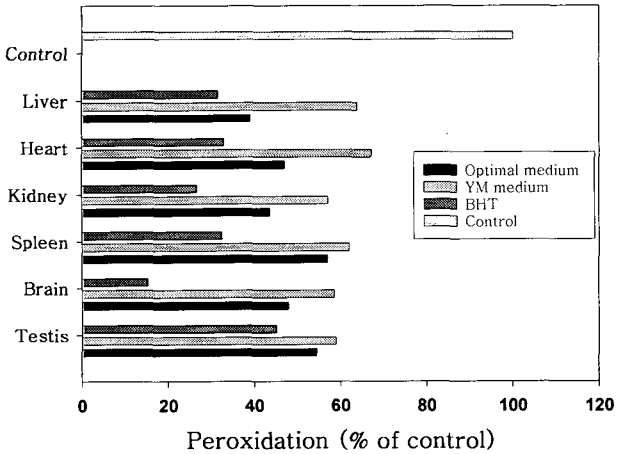

Fig. 2. Antioxidative activity of the cell-free extracts containing a high glutathione *Saccharomyces cerevisiae* FF-8 in organic microsomal system as measured by the ferric TBARS method. BHT : butylated hydroxytoluene (0.05%)

lipid peroxidation is commonly utilized for analysis. Polyunsaturated fatty acids such as linoleic acid, methyl linoleic acid, and arachidonic acid are typically used[4]. The antioxidative activities of the cell-free extracts of *S. cerevisiae* FF-8 were measured based on the inhibition of linoleic acid peroxidation by the ferric thiocyanate and TBA methods in this study. The TBA method was used for the measurement of lipid peoxidation, and an Fe^{2+}-ascorbate system was used for the catalysis of oxidation[4,10].

As shown in Fig. 3, the production of linoleic acid peroxide in the control reaction mixture increased rapidly from the first day and this increase continused for seven days in the ferric thiocyanate method. However, the cell free extracts of *S. cerevisiae* FF-8 showed a antioxidative effect of inhibiting lipid peroxidation compared with the control (Fig. 3). This antioxidative effect was more pronounced in the YM optimal medium cultured strain than that in the YM basal medium. BHT, which is one of the most widely used chemicals to retard oxidation, showed the highest antioxidative activity under this study conditions. The antioxidative activity of cell free extracts of *S. cerevisiae* FF-8 cultured in the optimal medium was almost equivalent to 0.05% BHT, which is the same concentration usually used in food. In the ferric TBA method, the production of linoleic acid peroxide in the control reaction mixture increased rapidly from the first day to the fourth day, which indicates the typical peroxidation curve of linoleic acid (Fig. 4). BHT

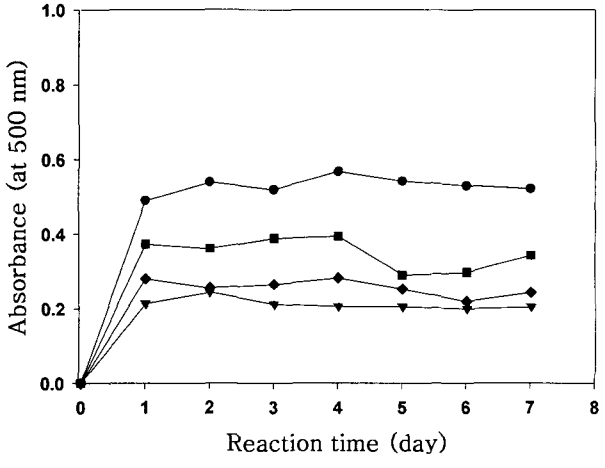

Fig. 3. Antioxidative activity of the cell-free extracts containing a high glutathione *Saccharomyces cerevisiae* FF-8 against the linoleic acid oxidation that is measured by the ferric thiocyanate method.
(-●-: Control, -▼- : BHT, -■- : YM basal medium, -◆- : YM optimal medium) BHT : butylated hydroxytoluene (0.05%)

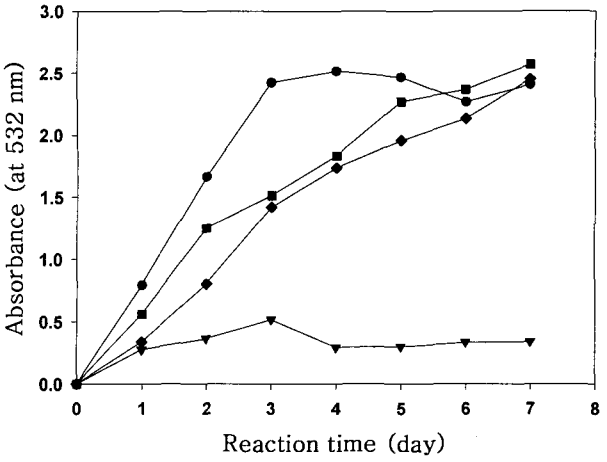

Fig. 4. Antioxidative activity of the cell-free extracts containing a high glutathione *Saccharomyces cerevisiae* FF-8 against the linoleic acid oxidation that is measured by the ferric TBA method.
(-●-: Control, -▼- : BHT, -■- : YM basal medium, -◆- : YM optimal medium) BHT : butylated hydroxytoluene (0.05%)

was the best antioxidant against peroxidation, and the cell free extracts of *S. cerevisiae* FF-8 cultured in the YM optimal medium had stronger antioxidative capacity than that of the YM basal medium against the peroxidation of linoleic acid under these conditions.

Based on present results, it can be concluded that the cell free of the intercellular glutathione containing by cultivating *S. cerevisiae* FF-8 in the optimal medium consisting of 3% glucose, 3% yeast extract, 0.06% KH_2PO_4, and 0.06% L-cysteine showing great free radical scaveging activity and lipid peroxidative inhibition effect against linoleic acid and tissues microsomal membraine lipids, could be used for early accessible microbial antioxidant sources.

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초록 : *In Vitro* 과산화지질에 미치는 glutathione 고함유 효모 *Saccharomyces cerevisiae* FF-8의 항산화효과

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항산화물질인 glutathione (γ-L-glutamyl-cysteinyl-glycine) 고함유 효모 *Saccharomyces cerevisiae* FF-8에서 생산된 glutathione을 함유한 세포 추출액의 항산화 활성을 *in vitro* 과산화지질 실험계인 DPPH(α,α'-diphenyl-β-picrylhydrazyl)법, linoleic acid를 이용한 ferric thiocyanate법과 TBA법 및 microsome 생체막 지질 과산화물 생성정도의 TBARS법으로 측정하였다. YM 최적 배지에서 생산된 glutathione 농도는 204 μg/mL로 YM 기본배지에서 생산된 glutathione 농도 74 μg/mL보다 2.76배 증가하였다. 본 실험에서는 YM 기본배지와 최적배지에서 *S. cerevisiae* FF-8이 생산하는 glutathione 함량에 따른 항산화 활성을 비교하였다. DPPH 측정법에서는 짙은 자색의 탈색되는 정도로 나타내는 전자 공여능이 glutathione 함량이 높은 최적 생산배지에서 *S. cerevisiae* FF-8가 생산하는 glutathione 고함유 세포 추출액에서 대조구인 0.05% BHT와 비슷한 수준으로 항산화 활성이 높게 나타났다. 각 조직 microsome을 이용한 생체막 지질 과산화 억제정도는 최적 생산배지에서 전체적으로 높게 나타났으며, 간장 60.98%, 신장 56.43%, 심장 52.91%, 뇌 52.13%, 고환 45.57% 및 비장 42.95% 순으로 나타났다. Linoleic acid 산화 실험계를 이용한 ferric thiocyanate법에서는 최적 생산배지가 반응 7일째까지 대조구에 비해 강한 항산화 활성을 보였으며, TBA법에서는 반응 5일째까지 최적 생산배지가 YM 배지보다는 높은 항산화 활성을 나타내었다. 이상의 결과에서 *Saccharomyces cerevisiae* FF-8 균주가 glutathione을 생산하는 최적배지 조건에서 생산된 glutathione 고함유 세포 추출액은 *in vitro* 항산화 실험계인 DPPH radical scavenging activity, ferric thiocyanate and TBARS 측정에서 항산화 활성을 나타내는 생리활성 성분을 지닌 것으로 나타나 천연 항산화제로서의 사용 가능성을 시사하였다.