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Bioremediation of water by removing manganese using *Saccharomyces cerevisiae* and isolation of high manganese-accumulating strain

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CHAPTER 1

INTRODUCTION

GENERAL INTRODUCTION

There are several anthropogenic activities largely responsible for the release of heavy metals into the environment, such as agriculture and sewage disposal, industries associated with the discharge of metals are mining, metallurgy, electroplating and other surface finishing industries, leather and tanning industries, energy production and pigment and battery manufacturing (Soares E. V. and Soares H. M. V. M, 2012). These industrial activities generate effluents containing various heavy metals at concentrations that mainly do not meet the wastewater limit discharge criteria; in these effluents, metals can be present at concentrations that may create a serious environmental hazard and a threat to water supplies (Soares E. V. and Soares H. M. V. M, 2012). The high toxicity of heavy metals, such as Mn, Cd, Pb, Hg, As, has been observed. Due to biomagnification and accumulation via food chain, the increasing risk of heavy metal concentration in the aquatic product occurred (Stankovic and Jovic, 2012; Whyte et al., 2009), causing significant threats to human health.

1.1 Mn contaminated in water

Groundwater aquifers in South and South-East Asia often contain high concentrations of dissolved iron (Fe) and manganese (Mn), which affect the taste and visual nature of the drinking water (Buschmann et al., 2007; Hug et al., 2008). Oxidation of soluble Mn(II) to form insoluble $MnO_{x(s)}$ precipitates in drinking water systems can cause aesthetic problems such as water discoloration and fouling, staining on plumbing fixtures, and consumer complaints (Dietrich, 2006; Whelton et al., 2007). The process using aerated sand filters for the removal of As, Fe, and Mn from

groundwater are well known. Homogeneous Mn oxidation by oxygen at neutral pH is relatively slow so that the formation of soluble Mn(III/IV) oxides occurs mainly as a result of heterogeneous and microbial Mn oxidation (Tebo et al., 2004; Learman et al., 2011; Luan et al., 2012). Although much is known about the abiotic processes contributing to the performance of sand filters and As, Fe, and Mn removal efficiency (Nitzsche et al., 2015) the composition of the microbial community and the potential contribution of different physiological groups of microorganisms to the removal of Mn have not been investigated for household aerated filters in Vietnam to date (Nitzche et al., 2015).

In Vietnam elevated arsenic (As), iron (Fe), and manganese (Mn) is found in aquifers of the Red River and the Mekong River deltas. Where Pleistocene sediments reach the surface in the upper Red River delta, groundwater Mn concentrations are largely over 0.6 – 3.4 mg L^{-1} (Buschmann et al., 2007; Hug et al., 2008). Public drinking water is pumped from the lower and older aquifer at depths of 150 – 250 m. The strongly reducing groundwater in the upper aquifers of the Red River and Mekong delta floodplain have high concentrations of As, Fe, and Mn (Hug et al., 2008).

The Red River delta of Vietnam has a population of some 11 million people. In rural areas, people changed the source of drinking water over 20 years ago, and nowadays they consume groundwater that is pumped through individual tube-wells (Berg et al., 2006). In the city of Hanoi, groundwater has been used for over 100 years for public water supply. Nowadays, due to the rapid population and economic growth, the increase of water demand is very urgent.

The Mekong delta has become one of the most densely populated and productive areas in Asia. Around 10,000 km^2 of the delta area is Cambodian and 52,000 km^2 is

Vietnamese territory. The number of people pumping contaminated groundwater for drinking has been estimated at 0.5-1 million (Berg et al., 2007). According to the data of Hug et al. (2008), 100% of groundwater samples in Bangladesh, Vietnam and Cambodia in three big river deltas (Bengal delta, Red river delta, and Mekong delta) has manganese concentrations higher ten to over sixty fold compared with United States Environmental Protection Agency (EPA) (1979), and to reach the appropriate to ensure consumer safety level (below 0.02 mg L^{-1}) this contamination rate up to over hundredfold. Buschmann et al. (2007) reported 75 samples (57%) of 131 well water samples that taken in Cambodia have manganese containing over 0.4 mg L^{-1} as WHO guideline.

Manganese (Mn) in drinking water is highly toxic to the living organisms and environment that is responsible for gastrointestinal accumulation (Henrik et al., 2004), low haemoglobin levels (Burgoa et al., 2001), neurotoxicity (Veliz et al., 2004), bad taste, cause color brown and pipe clogging. Mn concentration must be lower than 0.05 mg L^{-1} as a secondary contaminant according to the United States Environmental Protection Agency (EPA) (1979); however, a level of 0.02 mg L^{-1} is more appropriate to ensure consumer safety and minimize the potential for water discoloration. Although Mn is an essential element for living organisms, it becomes a toxic ion when present at high concentrations in cells (Au et al., 2008). Therefore, it is very imperative for removing heavy metals including Mn out of the underground water as well as water that is used for human activities. However, it is considered to be very difficult to removal heavy metal in water for the relatively low heavy metal concentrations and high concentrations of organic compounds (Ghimire et al., 2008). Unlike organic pollutants, metals and their salts are not degraded or destroyed, but rather they remain indefinitely

in the environment. Therefore, the release of heavy metals in the environment is a matter of concern and poses a different kind of challenge for remediation. In addition, there are many factors that inhibit the heavy metal removal such as high salt concentrations and low pH, which are frequently present in many kinds of wastewater.

Conventional physiochemical methods for heavy metal treatment such as chemical precipitation/neutralization, activated carbon adsorption, ion exchange resins, reverse osmosis, solvent extraction and electrochemical technologies have been used to treat effluents containing heavy metals. However, these processes are hardly applied as environmental friendly, fully efficient (precipitation/neutralization and electrochemical technologies) or present very high costs (membrane technologies and ion exchange resins), especially applied to large volumes of wastewater containing low metal concentration (1 – 100 mg L^{-1}) (Nguyen et al., 2013; Wang and Chen, 2009). Therefore, there is a great need for an alternative technique, which is environmentally friendly, efficient and economical.

1.2 Bioremediation methods

"Bioremediation is defined as the process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state, or to levels below concentration limits established by regulatory authorities". Bioremediation can occur on its own (natural attenuation or intrinsic bioremediation) or can be spurred on via the addition of fertilizers to increase the bioactivity within the medium. The advancements of bioremediation have also proven successful via the addition of matched microbe strains to the medium to enhance the resident microbe population's

ability to break down contaminants. Microorganisms used to perform the function of bioremediation are known as bio-remediators (Sharma, 2012).

To screen and select the most promising biomass with sufficiently high metal binding and metal accumulation capacity and selectivity for heavy metal ions are prerequisites for a full-scale bioremediation process, this is the first major challenge in the bioremediation field.

Yeast biomass is obtained in large amounts as a by-product of the brewing industry, and its use remains largely unexploited; therefore, its disposal is frequently an environmental problem (Ferreira et al., 2010). Due to the auto-aggregation properties of brewing yeast strains, they can be quickly and easily separated from the treated effluent; this intrinsic property avoids the use of cell immobilizing techniques or solid-liquid separation processes. After treating the effluent, the convenient management of the contaminated biomass and selective recovery of metals to ensure the minimization of waste production and low operating costs become important.

Saccharomyces cerevisiae (*S. cerevisiae*) is a unique biomaterial in bio-sorption/bioaccumulation research and application. It has long been and continues to be paid much attention. The advantages of *S. cerevisiae* as bio-material in metal biosorption or bioaccumulation that this yeast can easy to cultivate at large scale, it also easily grows at unsophisticated fermentation techniques and inexpensive growth media, and the yield of this biomass is also high (Kapoor and Viraraghavan, 1995). In addition, *S. cerevisiae* is usually used in the food and beverage industries. Its biomass can be cheap, safely and easily employed in rather substantial quantities, also as a by-product from fermentation processes. The biomass of *S. cerevisiae* as a by-product is easier to get from fermentation industry compared with other types of waste microbial biomass

because some microorganisms used in enzymatic industry and pharmaceutical industry are usually involved in the secret of their products. Moreover, *S. cerevisiae* biomass is generally regarded as safely; therefore, can be easily accepted by the public when applied practically. In addition, *S. cerevisiae* is also model organism to identify the mechanism of biosorption/bioaccumulation of metal ions, especially at the molecular level. Knowledge on the molecular biology of the yeasts is very helpful to identify the molecular mechanism of biosorption/bioaccumulation metal ions. These yeasts can also be easily manipulated genetically and morphologically to modify these yeasts more appropriate for various purposes of metal removal.

1.2.1 Saccharomyces cereivisiae in biosorption research

Generally, biosorption can be defined as the interaction of the sorbate (atom, molecule or ion) with the biosorbent (the solid surface of a biological matrix); this results in accumulation of the sorbate at the biosorbent interface and, consequently, a reduction of the sorbate concentration in the solution (Gadd, 2010). When referring to the control of heavy metal pollution, biosorption is the first step of metal removal. It is a rapid event (few minutes) and is brought about by live and dead cells (Brady and Duncan, 1994; Soares et al., 2002). It is metabolism independent and does not require the presence of a metabolizable energy source (Blackwell et al., 1995).

Biosorption consists of several mechanisms that quantitatively and qualitatively differ according to the species used, the origin of the biomass, and its processing. Metal sequestration follows complex mechanisms, mainly ion exchange, chelation, adsorption by physical forces; and ion entrapment in inner- and intrafibrillar capillaries and spaces

of the structural polysaccharide network as a result of the concentration gradient and diffusion through cell walls and membranes. There are several chemical groups that could attract and sequester the metals in biomass: acetamido groups of chitin, structural polysaccharides of fungi, amino and phosphate groups in nucleic acids, amino, sulfhydryl, and carboxyl groups in proteins, hydroxyls in polysaccharides, and mainly carboxyls and sulfates in the polysaccharides. However, it should be stressed that the presence of some functional group does not guarantee their accessibility for sorption, perhaps due to steric, conformational, or other barriers (Volesky and Holan, 1995).

A number of references have reported that *S. cerevisiae* can adsorb toxic metals, recover precious metals and clean radionuclides from aqueous solutions to various extents. Brady et al. (1994) reported that the yeast cells of *S. cerevisiae* that treated with hot alkali were capable accumulated a wide range of heavy metal cations, such as Fe(III), Cu(II), Cr(III), Hg(II), Pb(II), Cd(II), Co(II), Ag(I), Ni(II), and Fe(II). Autoclaving or the treatment of yeast cells with NaOH or ethanol has increased Cd(II) and Pb(II) biosorption (Goksungur et al., 2005). Ghorbani et al. (2008) reported that heat treatment followed by ethanol treatment has also increased Cd biosorption capacity by twofold. These enhanced capacities have been explained by an increase in the accessibility of the metal ions to the metal-binding sites on the biomass (Goksungur et al., 2005; Ghorbani et al., 2008).

The variety of metal ions such as lead, cadmium, copper, zinc, chromium, nickel, silver, iron, cobalt and uranium, etc. have been studied much more manganese, radium, selenium, precious metals. It should be noted that *S. cerevisiae* can distinguish different metals species based on their toxicity, such as mercury species (CH_3Hg and Hg(II)), antimony species (Sb(III) and Sb(V)). This kind of characteristic makes *S.*

cerevisiae not only useful for the removal or recovery of metal ions, but also for their analytical measurement (Madrid et al., 1995; Perez-Corona et al., 1997).

1.2.2 Saccharomyces cereivisiae in bioaccumulation research

In the case of living yeast cells, after the initial biosorption step, a second, metabolism-dependent step (bioaccumulation) can occur. The bioaccumulation of heavy metals is slower, occurs only in metabolically active cells and is influenced by the temperature and the presence of metabolic inhibitors (Blackwell et al., 1995). Bioaccumulation can be greatly enhanced in the presence of an external metabolisable energy source, such as glucose (Avery and Tobin, 1992) and could be desirable for heavy metal removal from effluents (Mapolelo and Torto, 2004; Stoll and Duncan, 1996). Recently, a *S. cerevisiae* mutant strain (*pmr1*Δ), which is hypersensitive to heavy metals due to increased uptake and has the enhanced ability to remove Mn(II), Cu(II) and Co(II) from synthetic effluents, compared with wild type, has been described (Ruta et al., 2010). Similarly, over-expression transporters that are responsible for the influx or arsenite (Fps1p or Hxt7p) in *S. cerevisiae* displayed an enhanced (three- to fourfold greater) accumulation of the contaminant (Shah et al., 2010).

The role of the vacuole in the detoxification of metal ions was investigated, and the results showed that vacuole-deficient strain displayed much higher sensitivity and the biosorption capacity for Zn, Mn, Co and Ni decreased (Ramsay and Gadd, 1997). However, no significant difference for Cd and Cu biosorption or sensitivity to both metal ions was observed between wild type and the mutant of *S. cerevisiae*. Gharieb and Gadd (1998) found that the vacuolar-lacking strains and the defective mutants of *S.*

cerevisiae display higher sensitivity to chromate and tellurite with a decrease in the cellular content of the each metal, whereas the tolerance to selenite increased with the cellular content of Se. Avery and Tobin (1992) also confirmed that Sr(II) accumulation occurs mainly in the vacuole of the living yeast cell of *S. cerevisiae*.

1.3 Metal bioaccumulation through growing cells

Bioaccumulation via growing cells is a potential technique for heavy metal removal from water. The heavy metals cannot only be biosorbed onto the cell surface but also pass into the cell across the cell membrane through the cell metabolic cycle (Brady and Duncan, 1994). The uptake mode that is independent of the biological metabolic cycle is known as "biosorption" or "passive uptake". The heavy metal can also pass into the cell across the cell membrane through the cell metabolic cycle. This mode of metal uptake is referred to as "active uptake". The metal uptake by both active and passive modes can be termed as "bioaccumulation" (Gadd, 1990). Most of the studies dealing with microbial metal remediation via growing cells describe the biphasic uptake of metals, i.e., the initial rapid phase of biosorption followed by slower, metabolism-dependent active uptake of metals (Donmez and Aksu, 1999; Garnham et al., 1992). The growing cells have unlimited capacities to split organo-metallic complexes, degrade organic compounds, as well as take up other inorganic ions such as nitrate, phosphate, ammonium (Malik, 2004).

Biosorption alone may not suffice for effective metal remediation. Mostly during the studies employing harvested biomass (dead/pretreated), metal is not taken up into the cells; rather, it is just adsorbed at the cell surface and, thus, only a small fraction of

bioaccumulation capacity is exploited (Torres et al., 1998). Under such situation, application of active and growing cells might be a better option due to their ability of self-replenishment, continuous metabolic uptake after physical adsorption, and the potential for optimization through a development of resistant species and cell surface modification (Wilde and Benemann, 1993). Further, the metals diffused into the cells during detoxification get bound to intracellular proteins or chelatins before being incorporated into vacuoles and other intracellular sites. These processes are often irreversible and ensure less risk of metal releasing back to the environment (Gekeler et al., 1988). Apart from this, using growing cells in bioremoval could avoid the need for a separate biomass production process, e.g. cultivation, harvesting, drying, processing, and storage prior to the use. In contrast to conventional chemo-physical and biosorptive methods, employment of active microorganisms may allow development of a single-stage process for removal of most of the pollutants present in industrial effluents. Growing cells have unlimited capacities to cleave organo-metallic complexes, degrade organic compounds, as well as take up other inorganic ions such as ammonium, nitrate, and phosphate. Further, dissolved and fine-dispersed metallic elements can also be removed via immobilization (Malik, 2004). Nevertheless, there are significant practical limitations to bio-uptake by living cell systems such as sensitivity of the system to extremes of pH, high metal/salt concentration and requirement of external metabolic energy (Donmez and Aksu, 2001). However, such challenges can be met via strain selection and exploitation of organic wastes as carbon substrates. In addition, metabolism-dependent uptake of heavy metal ions can be influenced by the presence of competing ions (Blackwell et al., 1995).

The isolation and selection of metal resistant strains shall be a crucial aspect to

overcome the prime constraint of employing living cell systems. In view of these facts, the applicability of growing cells in suitably configured bioreactor appears to offer promising biotechnology for combating heavy-metal pollution in the environment.

Several studies on the application of growing microbial cells for metal scavenging have been reported. However, in toxic metal removal applications, it is important to ensure that the growing cells can maintain a constant removal capacity after multiple bioaccumulation-desorption cycles, and a suitable method is required to optimize the essential operating conditions. The situation demands a multi-prong approach including strain isolation, cell development, and process development in order to make the ultimate process technically and economically viable (Malik, 2004).

1.4 Factors influencing heavy metal adsorption and/or accumulation

To understand the effects of factors, such as nutritional and environmental factors, on the quality and quantity of yeast biomass could help us to cultivate the desirable types of yeast cells on cheap nutrients and to use it in a suitable form for direct application (Goyal et al., 2003). Furthermore, metal accumulation and/or adsorption by yeast cells are affected by several physico-chemical properties of the solution, such as pH, the redox potential (Eh), the concentration of metal ions and complexing agents and the affinity of these agents for metal ions (Florence, 1983).

1.4.1 pH

Extremely of pH often decrease the accumulation of heavy metals in yeast cells. *S. cerevisiae* is able to accumulate heavy metals from solution between pH 5.0 and 9.0

(Brady and Duncan, 1994); moreover, pH values between 5.0 and 6.0 are optimal for Cu(II), Cd(II), Pb(II), Ni(II), Zn(II), and Cr(III) biosorption by yeast cells (Brady and Duncan, 1994; Ozer and Ozer, 2003; Parvathi and Nagendran, 2007; Zouboulis et al., 2001). The percentage of ionized groups on the yeast cell wall is extremely affected by the pH of the solution. In the case of living cells, the cell physiology and metabolism can be affected by the pH of the solution. At pH 6.0, the main chemical groups on the cell surface that can participate in the adsorption or accumulation of metal cations (carboxyl, phosphate, sulphydryl, hydroxyl and nitrogen-containing groups) are already completely (carboxylic acids) or partially (phosphate and amine groups) deprotonated. On the other hand, at low pH values, the functional groups on the yeast cell wall are progressively more protonated and metal accumulation decreases; moreover, the extremely pH in solution also affects the growth of yeast cells. Additionally, pH values can affect metal speciation. An increase in the pH can result in the precipitation of metal hydroxides. Consequently, less soluble amounts of metal will be able for accumulation in the yeast cells (Sheng et al., 2004).

For biosorption of heavy metal ions, pH is one of the most important factors that permit the efficiency of biosorption of yeast cells. The pH value of solution strongly influences not only the site dissociation of the surface of yeast cell wall but also the solution chemistry of heavy metals: hydrolysis, complexation, redox reactions, precipitation, the speciation and the biosorption availability of the heavy metals (Esposito et al., 2002). The biosorption capacity of metal ions can increase with increasing pH of the solution, but not in a linear relationship. The pH optimal of different biosorption system is different values. The optimal pH values for copper biosorption by *S. cerevisiae* is from 5.0 to 9.0, and this value is from 4.0 to 5.0 for

uranium (Volesky, 1990). Vianna et al. (2000) reported that the biosorption capacity of metal cations strongly depends on pH value. For the biosorption capacity of Cu, Cd and Zn at pH value 4.5 was much higher than at pH 2.5 and pH 3.5. Electrostatic attraction to negatively charged functional groups may be one of the specific biosorption mechanisms. Marques et al. (2000) researched the initial pH effects and the pH shift on the removal of Cu(II), Cd(II) and Pb(II) from unbuffered aqueous solution by non-viable cells of *S. cerevisiae*. A pH shift from 4.5 – 5.0 to a final value of 7.0 – 8.0 range was observed. The different removal mechanisms for each cation were suggested. For Cu(II), it was suggested that Cu(II) was removed by both the metal sorption and precipitation due to the pH shift occurred during the process, while Cd(II) removal was completely dependent on this pH shift. For Pb(II), it was totally and quickly removed by precipitation in the presence of the yeast suspension at pH 4.5.

The explanation of the pH shift in biosorption process and the pH effect is helpful to identify the mechanism of metal biosorption.

1.4.2 Temperature

For biosorption purpose, the temperature has also an influence on the biosorption of metal ions. Biosorption process is usually not operated at high temperature because of operation cost (Wang and Chen, 2006). The temperature from 5 – 40°C had a minor effect on the accumulation capacity of Cu(II), Cd(II) or Co(II) by free cells of *S. cerevisiae* in suspension (Brady and Duncan, 1994). Kapoor and Viraraghavan (1997) observed that biosorption capacity increased with decreasing temperature because adsorption reactions are normally release of heat. The maximum

equilibrium biosorption capacity for Pb(II), Ni(II), and Cr(VI) ions by the inactive yeast cells were reached at a temperature of 25°C in the range of 15 – 40°C. The decrease in capacity at higher temperature revealed that the processes of biosorption for these metal ions by *S. cerevisiae* are exothermic. Ozer and Ozer (2003) suggested that the decreased of biosorption capacity at a higher temperature may be due to the damage of active binding sites in the cell wall. However, Goyal et al. (2001) reported that the Cr(VI) biosorption of *S. cerevisiae* increases with increasing temperature in the range of 25 – 45°C, it is explained that higher temperature would lead to the higher affinity of sites for metal or binding sites on the yeast. The energy of the system facilitates Cr(VI) attachment on the cell surface to some extent. When the temperature is too high, there is a decrease in metal sorption due to distortion of some sites of the cell surface available for metal biosorption.

For bioaccumulation of living cells, temperature seems to be affected on to the growth of yeast cells and also to the metabolisms of cells. Usually, the optimum temperature for yeast growth maintains at 25 to 38°C (Jones and Hough, 1970). The extremely temperatures, such as lower temperature or higher temperature, also cause inhibition to the growth of yeasts; therefore, it is also affected metal ion accumulation of yeast cells.

1.4.3 Initial concentration of metal ions and biomass

For both cases of biosorption and bioaccumulation process, the increase of the biomass concentration of the biosorption or bioaccumulation system could result in increasing the sorption site interactions of cell walls. When the biomass concentration is

low, metal ions in the solution would not only be adsorbed to the surface of the biomass but also enter into intracellular part through facilitating the concentration gradient of metal ion and also metabolisms of cells. As a matter of fact, biosorptive and/or bioaccumulation of metal ions was reported to be related to the ratio of the concentration of initial metal ions to the concentration of biomass. Vasudevan et al. (2003) found that equilibrium uptake for Cd(II) ion by deactivated protonated yeast was directly proportional to the ratio of the initial metal ion concentration to the biomass.

1.4.4 Composition of cultural medium

Glucose is a carbon source widely used and acts as an important source of energy. For the bioaccumulation of living cells, the addition of glucose and other trace elements into a bioaccumulation system enhance the growth of yeast cells and facilitate metal bioaccumulation. Stoll and Duncan (1996) investigated that pretreatment of the yeast cells with glucose increased the amount of metal accumulated, while the direct addition of glucose into the yeast effluent mixed solution had no effect on the amount of metal accumulated. Avery and Tobin (1992) observed a different result that glucose was supplemented into medium just 5 min before the addition of Sr(II), the presence of glucose resulted in a stimulation of Sr(II) uptake due to metabolism-dependent intracellular Sr(II) accumulation, mainly in the vacuole. Mapolelo and Torto (2004) also observed that the pretreatment of the *S. cerevisiae* by using 10 – 20 mM glucose increased the removal efficiency for Cd(II), Cr(III), Cu(II), Pb(II) and Zn(II) by 30 – 40%, but the pretreatment by using 60 mM glucose decreased the removal of Cr(VI) by almost 50%. The mechanism of Cr(VI) uptake may be different from other metal ions.

The supplementation of cysteine, glucose, ammonium sulfate, phosphate and ammonium chloride in the fermentation media for the growth of *S. cerevisiae* increased the Cr(VI) uptake (Goyal et al., 2001). The best accumulation of Cr(VI) was obtained when the cells were grown in cysteine-supplemented medium, followed by glucose, phosphate, ammonium sulfate, ammonium chloride supplemented in media. Different nutrients clearly led to different functional groups in the corresponding cell surface. Cysteine inserts –S and –N ligands; glucose inserts –C ligands; ammonium inserts –N ligands; phosphate inserts –P ligands (Engl and Kunz, 1995). All of these, cysteine-rich media gave the highest adsorption capacity. The same results also obtained by Simmons and Singleton (1996). They obtained that addition of L-cysteine into the growth medium increased the bioaccumulation capacity for Ag, and also increased the content of protein and sulphydryl group in the freeze-dried and viable yeast cells. However, increasing the concentration of L-cysteine from 1 to 5 mM resulted in decreasing the cell numbers in the medium, in comparison with the control without L-cysteine supplemented.

1.4.5 Cell age

Cell age of biomass has also an influence on metal bioaccumulation. Usually, the cells at lag phase or early stages of growth have a higher bioaccumulation capacity for metal ions than that of stationary phase (Kapoor and Viraraghavan, 1997). Several yeast strains have been evaluated for bioaccumulation of metal ions under growing conditions. Donmez and Aksu (1999, 2001) reported that *Kluveromyces marxianus, Candida* spp. *and S. cerevisiae* removed 73 – 90% of Cu during their growth, although considerable differences in Cu accumulation capacity of different strains were found. They predicted that metabolically active cells from the exponential growth phase

probably contain highly active enzymes, some of which may be involved in complexing and binding the metal ions. Simmons and Singleton (1996) observed that the younger cell (24 h old) of an industrial strain of *S. cerevisiae* was almost accumulated Ag(I) concentration as high as twofold of that older cells (96 h old). They proved that intracellular components bind more Ag(I) than the cell wall. The addition of sulphur-containing amino acids into the medium improved the silver accumulation capacity by increasing cell protein levels and/or altering levels of sulphur-containing compounds within the cells.

1.5 Cell development

The treatment of metallic pollution could be enhanced by genetic manipulation of *S. cerevisiae* yeast cells because the complete genomic sequence of *S.cerevisiae* is available and the yeast is usually used as a model system in biology. *S. cerevisiae* can be easily manipulated genetically and morphologically, which are more appropriately for various purposes of metal removal. Therefore, yeast modifications have been performed to improve the efficiency of metal removal. However, because of the existence of legal and socio-political barriers, the using of genetically engineered yeast cells cannot be seen as a real alternative for removing metals in the real practical systems.

Another should practice ways of producing more resistant and efficient strain is through adaptation of the cells to progressively higher concentrations of metals which expected that adapted cells can have more metal uptake capacity and grow well in the presence of higher metal concentration than non-adapted cells. Donmez and Aksu (2001) adapted strains of *Candida* species (isolated from sewage) to Cu and Ni by serial

subcultures in Cu- and Ni-supplemented growth medium. Adapted cells could grow well in the presence of higher metal concentration while the non-adapted ones perished. Moreover, the specific metal uptake capacity and the metal removal (%) by adapted cells were higher than the non-adapted cells at all the concentrations tested. Although the authors could not elucidate the mechanism of adaptation, they implied constitutive synthesis of metallothionein/other copper-binding proteins or changes in the genetic makeup as one of the factors in adaptation. Further, in growing cell systems, availability of enough energy reserves facilitates active transport of metals for deposition into the vacuoles. Similarly, in the case of bacteria also, more tolerant strains have been produced via adaptation (Malik, 2004).

1.6 Content of this thesis

The present doctoral thesis is focused on setting for the results obtained in the studies of bioaccumulation of Mn from solution through growing cells to evaluate Mn-accumulation or Mn-absorption capacity of *S. cerevisiae*. Finding newly isolated-strain that has better ability to absorb or accumulate Mn, and optimize the growth of Mn resistant strain using some parameters such as temperature, pH, and the Mn absorption of the mutant strain. This work further reviews that use of *S. cerevisiae* and its Mn-resistant strain is an ideal for accumulation of Mn from water or wastewater as living cells material.

Beside Mn is one of the major metals causing serious problem to environmental, it also plays an important role in biological. Mn is required for the growth and survival, especially in adaptation to life in oxygen, for most living organisms (Horshurgh et al.,

2002). Although several recent studies have focused on trying to understand the biological chemistry of Mn, not much is known about ionic Mn *in vivo*, and the role of Mn as antioxidant remains to be resolved. Using the *S. cerevisiae* BY4741 as a model organism, I provide evidence for manganese ion supplemented in nutrient protected yeast cells more resistant to gamma-irradiation and others ROS sources, such as hydrogen peroxide and menadione. However, the Mn-accumulating strain *S. cerevisiae* IM3 that have much more Mn accumulation ability than the parental strain *S. cerevisiae* BY4741, was very sensitive to all sources of ROS generation. These results suggest for a new trend of Mn(II) function to scavenge ROS in yeast cells.

Although Mn(II) appears to be beneficial to yeasts for protecting yeast cells to ROS, the mode of action remains unclear. Manganese may work directly as a free radical scavenger, as it has been postulated to do so in unicellular organisms, or may work indirectly by upregulating several protective factors.

In the framework of this thesis, I would like to focus on bioremediation, especially bioaccumulation of Mn by *Saccharomyces cerevisiae* cells, attracting continuing attention for a developing satisfactory solution for the water decontamination in recent years. This study suggests a new strain of yeast that has significant Mn accumulation that is believed can become a promising strain for real water/wastewater treatment using living cells. The thesis is consists of 4 Chapters as follows:

In Chapter 1, I provided some overviews of Mn contaminant information, types of biomaterial using for bioremediation, methods for treatment of heavy metals in water/wastewater. Bioremediation using yeasts as biosorbent (dead cells/treated cells) or as bio-accumulator (living/alive cells) were also reviewed. Furthermore, purposes of this thesis were also addressed.

In Chapter 2, Mn accumulation of *Saccharomyces cerevisiae* BY4741 was examined and screening of high Mn-accumulating of variants was performed from the *Saccharomyces cerevisiae* strain. Based on the ability of Mn accumulation, a variant having a capacity of four to five-fold Mn accumulation than the parental strain was selected, suggesting that this variant may become a promising tool for Mn removal from water.

In Chapter 3, the function of Mn(II) ion in scavenging reactive oxygen species (ROS) within yeast cells was examined. It was shown that Mn(II) ion could act as an ROS scavenger to protect yeast cells against the ROS caused by ^{60}Co-gamma irradiation and other ROS sources such as hydrogen peroxide, menadione by using the yeast cells grown in nutrient medium supplemented with 0.5 mM of Mn(II). On the contrary, the isolated high Mn-accumulating variant showed a lower activity of catalase and superoxide dismutase and lower content of trehalose and was sensitive to ROS caused by ^{60}Co-gamma irradiation and other ROS sources such as hydrogen peroxide and menadione. These results suggest for a new trend of Mn(II) function to scavenge ROS complementing the other ROS scavenging activities of yeast cells.

In Chapter 4, the content of this thesis and perspectives of future research were summarized.

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CHAPTER 2

ISOLATION AND CHARACTERIZATION OF A VARIANT RESISTANT STRAIN OF *Saccharomyces cerevisiae*

2.1 INTRODUCTION

Many countries in Asia, such as Bangladesh, India, and Vietnam, where water for human use, including drinking water, comes from surface-water and under-ground sources, are facing issues of contamination with heavy metals such as arsenic, manganese, and iron that adversely affect the health of millions of people (Buschmann et al., 2008; Nitzche et al., 2015). In these countries, contaminated water is not only used for drinking but is also broadly used for irrigation of rice, especially during the dry season. Irrigation has led to the accumulation of heavy metals in paddy soil, and has consequently caused an increase in metal levels in rice grain. The widespread contamination of paddy field soils and increased heavy metal content in grain occurs because of extensive industrial and metal mining areas that cause heavy metal pollution in these countries. The most common inorganic pollutants in groundwater are iron and manganese. Manganese (Mn) concentration must be lower than 0.05 mg L^{-1} as a secondary contaminant according to the United States Environmental Protection Agency (EPA) (1979); however, a level of 0.02 mg L^{-1} is more appropriate to ensure consumer safety and minimize the potential for water discoloration. Although Mn is an essential element for living organisms, it becomes a toxic ion when present at high concentrations in cells (Au et al., 2008). In Vietnam, the two largest deltas, the Mekong and Red River deltas, play important roles in the lives of over 30 million Vietnamese. Based on a survey conducted in 2007 (Buschmann et al., 2008), Mn concentration in ground water in the Mekong River delta ranged from 0.01 mg to 14 mg L^{-1} (equivalent: 0.18×10^{-3} mM–0.25 mM Mn(II)). In the other largest river delta in Vietnam, the Red River delta located in northern Vietnam, average Mn concentration in underground water is over 1 mg L^{-1} (equivalent: 0.018 mM Mn(II)). Compared with the Mn guidelines of the World

Health Organization (WHO, 1998; Frisbie et al., 2012) of 0.4 mg L^{-1} (equivalent: 0.0072 mM Mn(II)), over 76% samples in this survey had a greater concentration of Mn. Recently, treatment techniques have mostly dealt with the other metals such as As, Cd, and Pb, but Mn has also become an important metal contaminant to be removed.

Although conventional methods for removing metal ions from aqueous solution, such as chemical precipitation, ion exchange, electrochemical treatment, membrane technologies, and adsorption on activated carbon, have been studied in detail, these methods are not efficient for treating large amounts of water or wastewater containing heavy metals at low concentrations (Wang and Chen, 2009; Schiewer and Patil, 2007). Therefore, an alternative technique is needed, which is safe, efficient, and economical. In addition to the advantage of low operating costs, a biological method is an ideal candidate for the treatment of high water volumes and low concentration of metal ions. Such a method may be able to decrease the concentration of heavy metal ions in solution from the ppt to the ppb level (Malik, 2004).

In recent decades, there has been increasing interest in using yeast and specific components of yeast cells as bio-sorbent materials. These applications have been based on new scientific evidence that suggest a role of yeast and yeast-derived products in modern systems. One of special interest is the use of *Saccharomyces cerevisiae* as biomaterial (Wang and Chen, 2009; Podgorskii et al., 2004). Using the yeast is not only economical but also flexibly adaptable to various conditions with genetic, non-pathogenic, and morphological manipulations that result in better raw biomaterials. Although there are some reports of heavy metal bioaccumulation by *S. cerevisiae* (Brady and Duncan, 1994a,b), these studies primarily focused on other heavy metals. Few studies have been conducted with Mn; thus, more research is needed to determine

bioaccumulation of Mn by growing cells.

Screening to determine suitable microbial strains, which are more efficient and adaptable to environmental stresses, is important to enhance the applicability of bioaccumulation of Mn using growing microorganisms as a biomaterial. There is also little information regarding new strains that may have a greater ability to accumulate Mn and be more applicable for water treatment using growing cells.

The objectives of this study were to: (1) evaluate Mn-accumulation or Mn-absorption capacity of *S. cerevisiae*, (2) isolate and characterize a mutant strain of *S. cerevisiae* that has better ability to absorb or accumulate Mn than the parental strain. The growth of Mn resistant strain was optimized using some parameters such as temperature, pH, and the Mn absorption of mutant strains has been investigated to check applicability to practical water or wastewater treatments.

2.2 MATERIALS AND METHODS

2.2.1 Strains and cultivation

S. cerevisiae strains used were BY4741 (*MATa his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*), obtained from Open Biosystems (Lafayette, Co., USA), and its mutant IM3, spontaneously grown on solid yeast extract peptone dextrose (YPD) medium with highly concentrated Mn. The YPD media was composed of 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) D-glucose (pH: 5.8 – 6.0). If solid medium was used, 1.5% (w/v) agar was added.

Sterilized Mn stock solution (Mn-solution) was prepared by dissolving 0.5 g

manganese chloride with tetrahydride in 10 mL of water (final Mn(II) concentration was 0.25 M). Media with manganese ions (Mn(II)) were created by addition of diluted Mn-solution.

S. cerevisiae strains were pre-cultivated in 5 mL YPD liquid media for 24 h with shaking at 120 rpm at 30°C. Mid-exponential phase cells (approximately 1.0×10^6 cells ml^{-1}) were harvested, washed twice with 0.9% sodium chloride (saline solution), and re-inoculated in 5 mL fresh YPD liquid media with shaking at 120 rpm at 30°C.

2.2.2 Assay for the effect of Mn concentration on yeast growth

Different Mn(II) concentrations of 0, 1, 5, 10, and 15 mM were added in liquid YPD medium contained initial approximately 1.0×10^6 cells ml^{-1} at 30°C in a rotary shaking incubator (120 rpm). After culturing for various periods, yeast growth was monitored by measuring the absorbance of light of 660-nm wavelength, and the number of yeasts cells was determined by Flow Cytometry using the BACTANA (Sysmex, Japan).

2.2.3 Measurement of Mn within cells

One milliliter of yeast cell suspension was collected, placed into micro tubes 1.5 mL, centrifuged at 4000 rpm for 5 min at 4°C, washed three times with distilled water, and digested by 6 M pure nitric acid for about 1 h at 90°C. Following adequate dilution with distilled water, the Mn concentrations in samples were analyzed using the atomic absorption spectrophotometer Z-8240 (Hitachi Co., Tokyo, Japan), with reference to the appropriate standard. All the samples were analyzed at least three times and the data

reported are means ± standard deviations.

2.2.4 Heating, irradiation, and starvation treatments for checking Mn adsorption of dead Saccharomyces cerevisiae cells

After being pre-cultured in YPD liquids, approximately $5x10^6$ cells mL^{-1} were collected and killed by heating at 100°C for 15 min or by ^{60}Co gamma-irradiation at 10 kGy. Cell viability was checked by no colony formation in YPD-agar plates including the cell suspensions after treatment by heating and irradiation after 3-4-day incubation at 30°C. For starvation treatment, cells were collected by centrifugation and directly used without killing.

Cell suspensions were supplemented in 5 ml sterilized water with Mn at a concentration of 10 mM and incubated with shaking at 120 rpm at 30°C for 24 h. Yeast cells were collected and washed three times with distilled water before being analyzed by AAS to determined Mn adsorption concentrations.

2.2.5 Isolation of Mn resistant mutants

S. cerevisiae strain BY4741 was cultured in YPD liquid medium with 0.01 M Mn(II) for 48 h, washed once with saline, and spread on a YPD agar plate after adequate dilutions. After being cultured for 3–4 days at 30°C, approximately 20 contingent colonies were selected and inoculated on YPD plates with 10 mM Mn(II), which were used as a “master-plate”. Colonies that survived on the master-plate were selected and re-inoculated in YPD liquids with 10 mM Mn(II) for 24 h. Next, yeast cells were spread out in YPD plates and 20 random colonies were selected to inoculate YPD plates with

10 mM Mn(II). The same process was repeated several times, and well-growing strains were isolated as the Mn-resistant mutants.

2.2.6 Effect of temperature on growth of Mn resistant mutant strain

Culture media of 5-mL fresh YPD supplemented with 10 mM Mn and without Mn supplemented (as control) were cultivated at different temperatures of 14^{o}C, 30^{o}C, 37^{o}C, and 42^{o}C to determine the effect of temperature. Growth profiles at OD 660 nm were determined. Manganese accumulation of yeasts at different temperatures was analyzed after 24 h of cultivation.

2.2.7 Effect of pH on the growth and Mn accumulation of Mn resistant mutant strain

The YPD medium contained a volume of 1/100 pre-cultured was adjusted to pH 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 using 0.1 N NaOH or 0.1 N HCl aqueous solution supplemented with 10 mM Mn(II). Fresh YPD medium without Mn supplement was used as control sample. The growth curves of BY4741 and IM3 at these conditions were recorded by OD 660 nm every 3 h.

2.3 RESULTS

2.3.1 Effect of Mn(II) on the growth of yeast cells Saccharomyces cerevisiae BY4741

The growth profiles of *S. cerevisiae* strain BY4741 are shown in Fig. 2.1, with cultures in YPD with various Mn(II) concentrations. The growth of BY4741 was not influenced until Mn concentration reached 1 mM. Nevertheless, the growth of BY4741 decreased at Mn concentration greater than 3 mM (data not shown), and very little growth occurred at Mn(II) concentrations higher than 10 mM (Fig. 2.1). Yeast cells need a long lag phase showed at Mn concentrations of 5 mM and higher, whereas lower Mn(II)-supplemented samples and the control had only approximately a 6-h lag-phase (exponential phase). Manganese concentrations in media higher than 10mM showed the growth inhibition to *S. cerevisiae* BY4741.

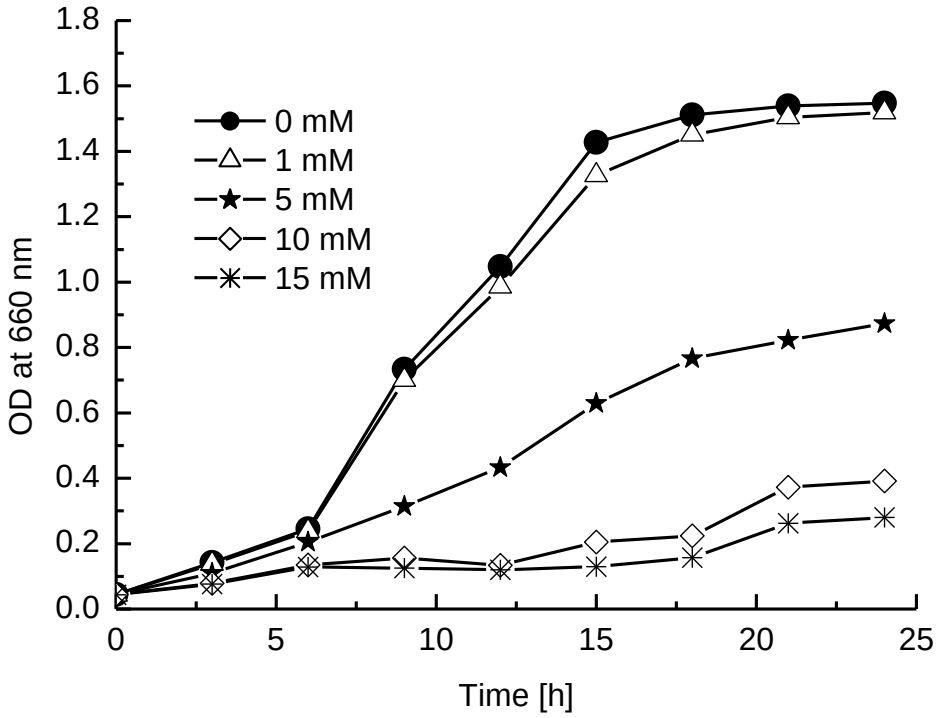

FIG. 2.1: Growth curves of *S. cerevisiae* BY4741 in YPD media supplemented with various Mn(II) concentrations. Initial cell number was adjusted to 1×10^6 cells mL^{-1} (equivalent $OD_{660} \approx 0.05$).

2.3.2 Manganese adsorption and/or accumulation in Saccharomyces cerevisiae BY4741

Samples containing 5×10^6 cells mL^{-1} of yeast were incubated in YPD with Mn(II) solutions in which the final concentrations were 0, 1, 5, 10, and 15 mM. After 24 h of incubation at 30°C with shaking at 120 rpm, the intracellular Mn(II) concentrations of all cell samples were analyzed (Fig. 2.2).

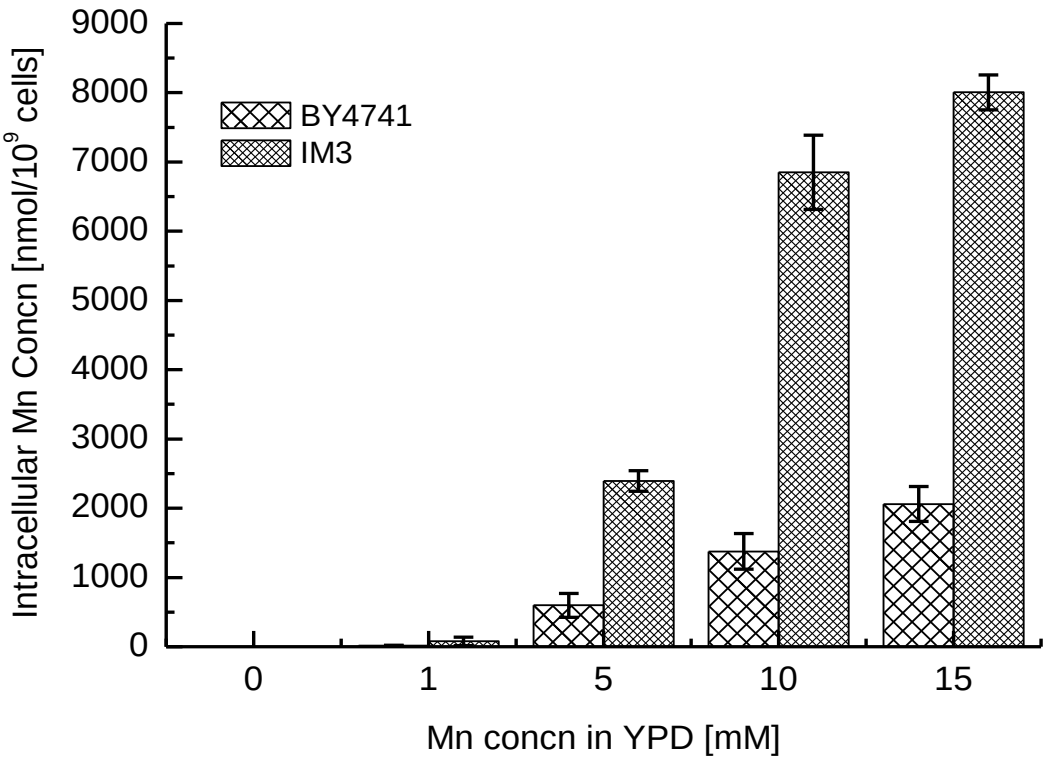

FIG. 2.2: Mn accumulation of the variant strain *S. cerevisiae* IM3 compared with parental strain *S. cerevisiae* BY4741

In *S. cerevisiae* BY4741, Mn concentration within the cell gradually increased with increasing Mn(II) concentrations in the culture media and reach over 2 μmol/10^9 cells after growth in 15 mM Mn(II) containing media. Interestingly, yeast cells at the exponential phase accumulated more Mn than did yeast cells at the stationary phase (data not shown). Thus, yeast cells may actively accumulate Mn ions from the environment, and the magnitude of Mn accumulation depends on the growth phase of the yeast cells.

2.3.3 Manganese adsorption of yeast cells inactivated by heating and irradiation, and yeast cells in water without nutrients

Manganese concentrations of yeast cells in high concentrations of manganese supplementation (10 mM) are showed in Table 2.1. Manganese adsorption was only 0.34 μmol/10^9cells for yeast grown in water alone, and 0.73 μmol/10^9cells for yeast inactivated by irradiation. No Mn was detected in cells inactivated by heating. Compared with growing yeast cells, Mn accumulation of yeast cells inactivated by heating or irradiation, or incubated in water without nutrients, was very low, suggesting that the Mn accumulation capacity of yeast cells is dependent on the activities of yeast.

2.3.4 Isolation and Mn accumulation of a Mn-resistant strain

Following the isolation process, some colonies were isolated resistant with 10 mM Mn(II) in the media. One colony among them, colony number 3, showed highest growth and greater Mn absorption ability than the parental strain BY4741. The strain was named *Saccharomyces cerevisiae* IM3.

To determine whether this was a true mutant strain or the yeast simply adapted with the cultures, *S. cerevisiae* IM3 was cultured several times in YPD media without supplemental Mn. After that, it was inoculated in YPD media supplemented with 10 mM Mn(II) and the OD value of the culture was 1.2 after 24 h of culturing time.

The growth profiles of IM3 under various supplemental Mn concentrations in YPD are shown in Fig. 2.3. Growth of the parental strain BY4741 appeared to be limited at 10 mM Mn. On the other hand, the growth of IM3 increased at concentrations greater than 15 mM Mn(II) (Fig. 2.3), and the Mn accumulation of IM3 was also 4-fold

to 5-fold higher compared with that of the parental strain (Fig.2.2). These results suggest that IM3 is more resistant and has a greater ability to accumulate Mn than its parental strain BY4741 in the presence of Mn(II) ions.

To characterize Mn-absorption capacity of IM3 compared with the parental strain BY4741, IM3 viable cells and the cells inactivated by heating or ^{60}Co gamma-irradiation were incubated in Mn-containing water without nutrients,. The same trend of the Mn concentrations as seen with the parental strain was obtained as shown in Table 2.1, suggesting that the IM3 cells has the same characteristics as their parent BY4741 cells in accumulating Mn.

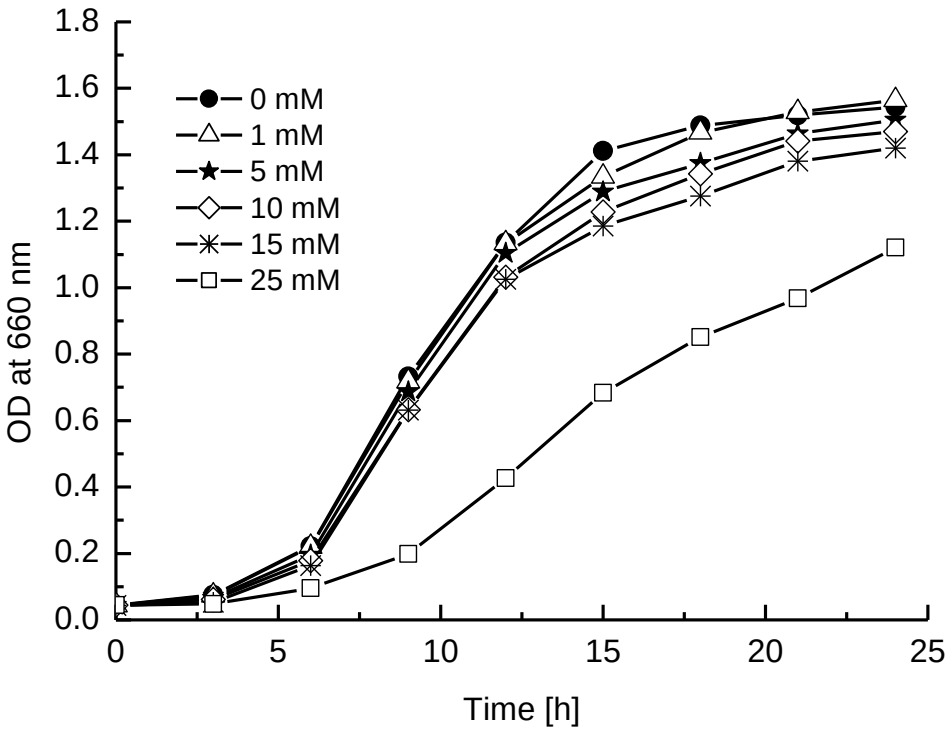

FIG. 2.3. Growth curves of *S. cerevisiae* IM3 in YPD media supplemented with various Mn(II) concentrations. Initial cell number was adjusted to 1×10^6 cells mL^{-1} (equivalent $OD_{660} \approx 0.05$).

TABLE 2.1. Manganese concentration in cells of *S. cerevisiae* BY4741 and IM3 affected by the treatments of starvation with and without heating and irradiation as pretreatments

Strain	Treatment	Incubation*	Mn concn (μmol/10^9 cells)
BY4741	None	YPD	1.38±0.26**
	None	Water	0.34±0.01
	Irradiation	Water	0.73±0.05
	Heating	Water	ND***
IM3	None	YPD	6.85±0.54
	None	Water	0.05±0.01
	Irradiation	Water	0.58±0.02
	Heating	Water	ND

* Cells were incubated at 30°C in the presence of 10mM $MnCl_2$

**SD.

***Not detected (<0.01)

2.3.5 Effect of temperature on growth of Mn resistant mutant

The 1/100 initial volume of each strain was incubated in YPD, and the YPD was supplemented at a concentration of 10 mM Mn(II). After 24 h incubation with shaking at 120 rpm at different temperatures, 14°C, 30°C, 37°C, and 42°C, the growth curves of (Fig. 2.4) and intracellular Mn concentrations of both strains were analyzed (Table 2.2).

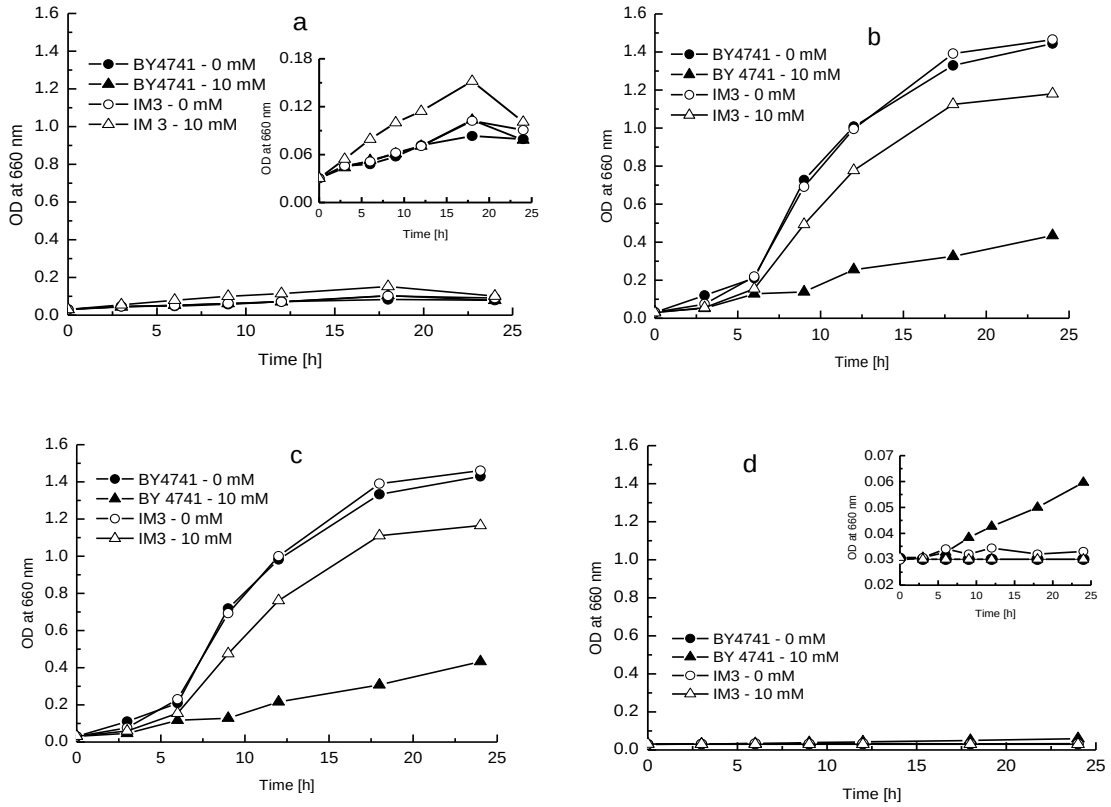

FIG. 2.4. Effect of temperature on the growth of *S. cerevisiae* BY4741 (closed symbols) and IM3 (open symbols) in YPD medium at pH 6.0 supplemented with (triangles) or without 10 mM Mn(II) (circles). Yeast cells were cultivated at temperature of 14 (a), 30 (b), 37 (c), and 42ºC (d) with shaking 120 rpm. Inlets are figures with enlarged scales

TABLE 2.2. Mn accumulation of yeast strains cultured in YPD media supplemented with 10 mM Mn for 24 h at different temperatures

Temperature (°C)	Strain	Mn concn (μmol/10^9cells)
30	BY4741	1.46±0.02
	IM3	6.16±0.03
37	BY4741	1.23±0.01
	IM3	5.82±0.02

For both strains, temperatures of 30°C and 37°C appeared to be the optimal temperatures for growth (Fig. 2.4-b and 2.4-c),. The growth of both strains was limited by the extremely high temperature of 42°C (Fig. 2.4-d), and the low temperature of 14°C limited growth of yeast cells. However, yeast cells still grew at a very low rate, especially when Mn was supplemented in the YPD media (Fig. 2.4-a).

Although 37°C was the optimum temperature for the growth of both strains, Mn accumulation of both strains stay at similar level at 30°C (Table 2.2).

2.3.6 Effect of pH on the growth and Mn accumulation of Mn resistant mutant

The YPD liquid medium was a neutral to slightly acidic medium for yeast cells. To determine the range of optimum pH for growth and accumulation of Mn, yeast cells of both strains BY4741 and IM3 were inoculated in YPD media, the YPD media was supplemented with 10 mM Mn(II), and pH values were adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 by 0.1 N NaOH or 0.1 N HCl aqueous solutions before the culture started.

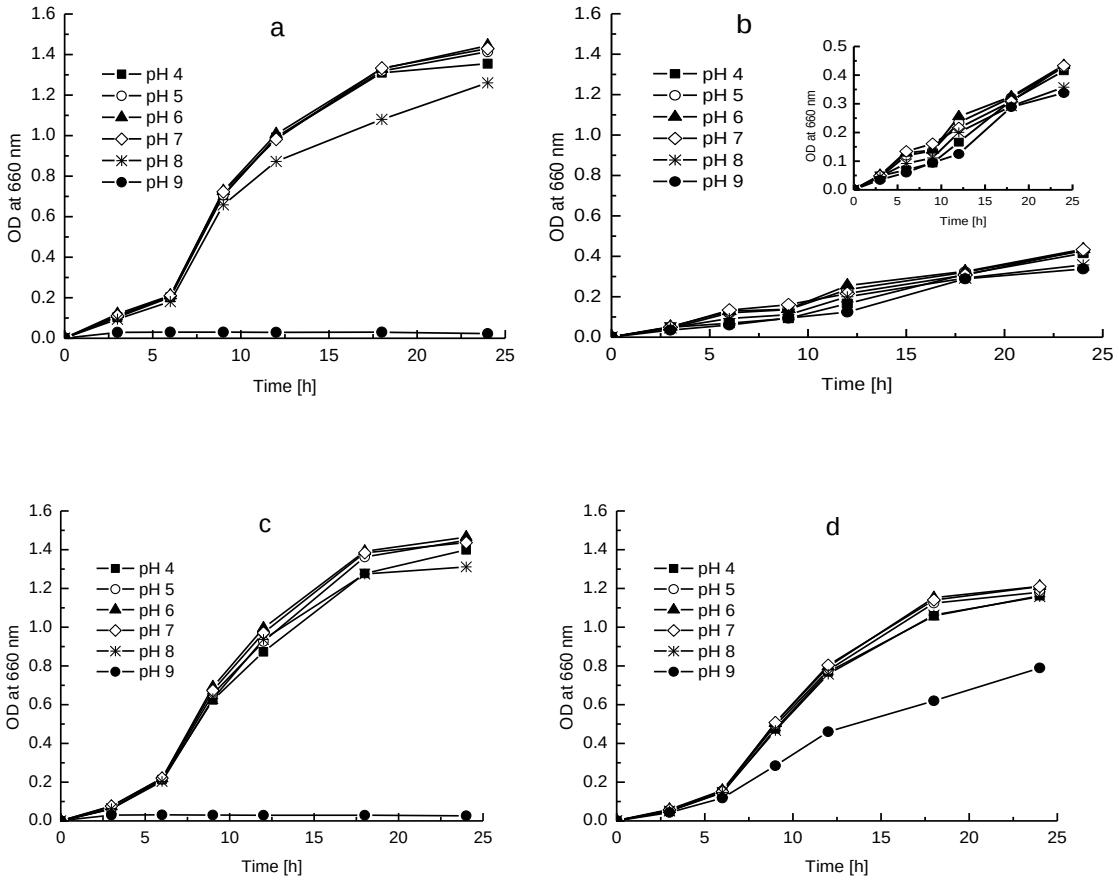

FIG. 2.5. Effect of pH on the growth of *S. cerevisiae* BY4741 (a, b) and IM3 (c, d) in YPD medium supplemented with (b, d) or without 10 mM Mn(II) (a, c). Yeast cells were cultivated at 30ºC with shaking 120 rpm. Inlet is a figure with enlarged scales

As showed in Figure 2.5, growth of yeast cells of both strains in the acidic or alkaline zone was slightly lower than in the neutral pH range. Yeast cells of both strains grew normally in a range of pH of 4–8, although a pH of 9.0 limited growth of yeast strains (Fig 2.5-a, 2.5-c). Interestingly, in YPD media supplemented at 10 mM Mn(II), even at pH 9.0, yeast cells of both strains grew at a higher rate compared with normal conditions, even though 10 mM Mn(II) was detrimental to growth of BY4741 (Fig 2.5-b, 2.5-d). For IM3 in media supplemented to 10 mM Mn(II), the Mn ion may help yeast cells adjust the pH inside and outside of the cells to adapt to the alkaline condition. Yeast cells still grew, albeit at a slightly lower rate compared with other pH values (Fig. 2.5-d).

Table 2.3 shows the Mn accumulation of BY4741 and IM3 at different pH values. Manganese-accumulation ability of both strains exhibited the same trend. Manganese absorption of both strains was highest at pH 6.0, and became lower when pH became more acidic or alkaline.

TABLE 2.3. Manganese accumulation of yeast strains cultured in YPD media supplemented with 10 mM Mn for 24 h at different pH values

pH value	Manganese concentration ($\mu mol/10^9 cells$)	
	BY4741	**IM3**
4	0.75±0.01	2.67±0.01
5	0.97±0.01	3.46±0.01
6	1.31±0.02	6.55±0.01
7	1.17±0.01	5.78±0.01
8	1.00±0.02	6.47±0.01
9	0.81±0.02	2.85±0.02

2.4 DISCUSSION

Blackwell et al. (1998) studied *S. cerevisiae* NCYC 1383 grown in YPD media supplemented with a range of Mn(II) concentrations as $Mn(NO_3)_2$, and reported that intracellular Mn after cultivation for 16 h was absorbed highest in the 20 mM Mn(II)

supplemented media, with a Mn concentration value of 1.7 μmol/10^9cells. Intracellular Mn concentration gradually increased as the Mn concentration of the culture increased but the cell growth was retarded at Mn concentrations higher than 4 mM (Blackwell et al., 1998). Our present study with BY 4741 also indicated the same trend and the isolated variant IM3 showed the 4-fold to 5-fold higher Mn accumulation capacity and the significant tolerance against the same level of the Mn concentrations employed for the parental strain. This new variant may be promissive to control Mn in various water treatments.

Microorganisms have several mechanisms of intake or accumulation and storage of metal ions. The accumulation can also be accompanied with sorption of ions and their binding by polymers or function groups on the cell wall. Our study clearly showed that both S. cerevisiae BY4741 and IM3 had little capacity for Mn adsorption, indicating that Mn ions move into the yeast cells by the mechanism of chemiosmosis gradient (Rosenfeld and Culotta, 2012). The most common phenomenon is specific active transport of some ions into the cells through specific pathways or ions channels (Reddi et al., 2009).

Several authors have reported that vacuoles function in Mn storage and detoxification. As such, the vacuolar function effect on Mn tolerance is not surprising (Lichko et al., 1980; Culotta et al., 2005). Uptake of several metals ion such as Mg(II), Fe(II), Mn(II) into the vacuole is known to depend on the activity of the V-ATPase and the proton gradient it establishes across the vacuolar membrane (Okorokov et al., 1976; Li et al., 2001). If, based on these ideas, Mn accumulation of *S. cerevisiae* may be enhanced by function of chemiosmosis gradient of Mn inside and outside the yeast cell, Mn may be more vigorously accumulated by *S. cerevisiae*. Park et al. (2003) reported

that the thicker mannan layer and the larger specific surface layer of *S. cerevisiae* ATCC 834 seems to benefit a larger cadmium uptake capacity. Avery and Tobin (1992) confirmed that Sr(II) accumulation occur mainly in the vacuole of the living yeast cell of *S. cerevisiae.* Based on these papers, we suppose the superior Mn(II) accumulation ability of the IM3 to be caused by such a difference of cell structure.

Our present study also suggests that Mn-absorption mechanisms of *S. cerevisiae* BY4741 and the variant strain *S. cerevisiae* IM3 may be closely related to the same cell activities during their life cycle because the Mn-accumulating activity is similar throughout their growth phases and no Mn accumulation is observed without growth (i.e., cells are killed) by inactivation by heating or ^{60}Co gamma-irradiation, and starvation.

Based on these facts, the development of systems using microbial biomass as a continuous system to remove metals based only on bio-adsorptive/adsorption appears to be impossible or uneconomic. However, application of growing cells and active cells for continuous systems not only at the laboratory scale, but also for real treatment systems, might be a better candidate because of the abilities of living cells, such as continuous metabolic absorption of metals and self-replenishment. Furthermore, the metals that penetrated into the cells were detoxified during metabolic activities of cells that combined them to intracellular proteins or chelatins before being integrated into vacuoles and other intracellular organelles. This results in detoxified metals and often reduces the risk of metals returning to the environment (Gekeler et al., 1998). In addition, use of growing cultures may allow for the exploitation of the process for the removal of many kinds of pollutants. Living cells have almost unlimited capacities to degrade organic materials, even organic-metallic complexes, and uptake inorganic ions

such as ammonium, nitrate, and phosphate. Although several studies on application of living cells for treatment of metal ions have been conducted, the practical application of growing cells still face factors, such as extreme pH, high salt concentration, and energy requirements (Donmez and Aksu, 2001). Thus, finding suitable methods and microbial strains of growing cells that can maintain their accumulation capacity after multiple bio-absorption-desorption cycles, have high metal bioaccumulation, and grow well under environmental stress is required for practical application.

In conclusion, growth property of *S. cerevisiae* IM3 in the YPD medium without Mn supplementation was the same as the parental strain BY4741, but, under Mn supplementation conditions, *S. cerevisiae* IM3 exhibited four- to five-fold the Mn accumulation than the parental strain. This suggests that this strain could be a good candidate for practical application using living cells

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CHAPTER 3

THE ROLE OF INTRACELLULAR MANGANESE IN YEAST AGAINST ROS CAUSED BY GAMMA-IRRADIATION

3.1 INTRODUCTION

Ionizing radiation is an extremely stress to all the living organisms through the action of reactive oxygen species (ROS) formed by the radiolysis of water in which generating hydroxyl radicals (OH^{-}), superoxide (O_2^{-}) and hydrogen peroxide (H_2O_2) that are caused indirect effects to the cell's macromolecules or ionizing radiation can directly damage all types of molecules (e.g., DNA, proteins, lipids) (Daly, 2009; Riley, 1994). It is now indicated that proteins are also major targets of radiation damage and an essential process for organism survival from ionizing radiation is that proteins are protected from oxidation (Du and Gebicki, 2004; Daly et al., 2007).

Intracellular manganese has long been acknowledged to extinguish oxidative stress in a diverse of organisms. Mn accumulation in cells seems beneficially extend to protect active sites of enzymes from oxidative damage. Replacement of Fe(II) and other divalent cations (Mg(II), Cu(II)...) with Mn(II) ion as the mononuclear cofactor in enzymes would thus protect active sites from oxidative damage. Several enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) are antioxidant enzymes that act as a major function against reactive oxygen species (ROS) which are causing oxidative damage and may possibly result in cell death. Manganese is an essential transition metal that is required for the growth and survival of most if not all, living organisms (Whittaker, 2010; Horsbugh et al., 2002). Although manganese is most commonly associated with its role as a catalytic and/or structural protein cofactor (Robinson, 1998; Culotta et al., 2006), the majority of manganese is thought to be present as low-molecular-weight Mn(II) ion complexes that, among other functions, can act independently of proteins to either defend against or promote oxidative stress and disease (Daly, 2009; Reddi, 2009).

Experiments *in vivo* that mainly studied in bacteria have shown that Mn can act catalytically as a scavenger of either superoxide or hydrogen peroxide that is likely to involve redox reactions between the Mn(II) and Mn(III) states (Archibald and Fridovich, 1981). Although several recent studies have focused on trying to understand the biological chemistry of Mn, not much is known about ionic Mn *in vivo* and the role of Mn as antioxidant remains to be resolved.

The result comes from Sanchez et al. (2005) that ionic manganese at high levels is beneficial to *sod1Δ* yeast which strain is knockouts CuZnSOD. In other words, yeast strains were grown in media containing millimolar levels of manganese rescues all phenotypes of the compromised knockout strain even in the absence of MnSOD, suggesting that the manganese rescue is also independent of MnSOD activity in yeast. Sanchez et al. (2005) only proved that manganese ion at millimolar level rescues all strain, however, there has been no reports about manganese concentration level in yeast. Is this possible that the normally high levels of Mn inside the cell play a significant role in their resistance?

Strong lines of evidence from different laboratories have thus converged on the conclusion that the accumulation of Mn(II) ion with orthophosphate (Pi) together with the certain organic metabolites represents a widespread strategy for combating oxidative stress (Barnes et al., 2008; McNaughton et al., 2010; Daly et al., 2010). Intracellular Mn(II) ion speciation within *Saccharomyces cerevisiae* has also recently been probed through measurements of ^{1}H and ^{31}P electron-nuclear double resonance signal intensities, which support an important role for the orthophosphate (Pi) complex of Mn(II) ion in cellular resistance to oxidative stress in this eukaryote (McNaughton et al., 2010).

To our knowledge, there is very little research on the effects of Mn and other divalent cation levels on yeast cells resistance to ROS generation, especially caused by gamma irradiation. To uncover if manganese has a pro/antioxidant property *in vivo*, we provided varying levels of Mn to the growth medium to see how Mn supplementation effects yeast development. Some well known antioxidant enzymes such as superoxide dismutase (SODs), catalase (CAT) and trehalose content of *S. cerevisiae* were also determined when Mn-supplemented in medium to see their function protect yeast cells to gamma irradiation.

Using the *S. cerevisiae* BY4741 as a model organism, we provide evidence for manganese ion supplemented in nutrient protected yeast cells more resistant to gamma-radiation and manganese ion concentration levels supplemented in nutrient in which have not affected the growth of yeast help yeast cells more tolerant to oxidative stress caused by gamma-irradiation. Even as a comparative metal ion to iron in catalase, manganese ion has its abilities to help yeast cells more tolerant to gamma-irradiation than catalase activity. Superoxide dismutase enzymes (SODs) were also provided oxidative stress resistance to protect yeast cells to ROS caused by gamma irradiation. However, using the isolation strain *S. cerevisiae* IM3 that isolated from 10 mM Mn(II) ion supplemented in medium, which have four to five-fold Mn accumulation than the parental strain *S. cerevisiae* BY4741, the isolated high Mn-accumulating variant showed lower activity of catalase and superoxide dismutase and lower content of trehalose and was sensitive to ROS caused by ^{60}Co-gamma irradiation and other ROS sources such as hydrogen peroxide and menadione. According to these results, it was suggested for a new trend of Mn(II) ion function to scavenge ROS collaborating with other ROS scavenging functions in yeast cells.

3.2 MATERIALS AND METHODS

3.2.1 Strains and Cultivation

S. cerevisiae strains used were BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), obtained from Open Biosystems (Lafayette, Co., USA), and its mutant IM3, spontaneously grown on solid yeast extract peptone dextrose (YPD) medium with highly concentrated Mn. The GYP media was composed of 0.5% (w/v) yeast extract, 0.5% (w/v) peptone, and 2% (w/v) D-glucose (pH: 5.8 – 6.0). If solid medium was used, 1.5% (w/v) agar was added.

Sterilized Mn stock solution (Mn-solution) was prepared by dissolving 0.5 g manganese chloride with tetrahydride in 10 mL of water (final Mn(II) concentration was 0.25 M). Media with manganese ions (Mn(II)) were created by addition of diluted Mn-solution.

S. cerevisiae strains were pre-cultivated in 5 mL YPD liquid media for 24 h with shaking at 120 rpm at 30°C. Mid-exponential phase cells (approximately 1.0×10^6 cells ml^{-1}) were harvested, washed twice with 0.9% sodium chloride (saline solution), and re-inoculated in 5 mL fresh YPD liquid media with shaking at 120 rpm at 30°C.

3.2.2 Growth profiles of yeast strains in media supplemented with Mn(II) ion concentrations

S. cerevisiae BY4741 and IM3 cells were pre-culture in 5ml GYP medium for 24h with shaking at 300 rpm, 30°C. Approximately 1/100 volume was transferred to new 5ml GYP media supplemented with various concentrations of Mn(II) ion and incubated at 30°C with shaking 120 rpm.

The density of suspension will be recorded under OD 660 nm every 6 hours.

To know how much manganese can be absorbed in the cell, 1ml suspension of the culture before irradiated was collected in the Eppendorf tube. These samples will be washed with distilled water 3 times and dried 1 hour at 50^{o}C before digested by HNO_3 at 90^{o}C for 1 hour. After diluted with distilled water to 50ml, 0.02ml of the sample will be analyzed by Atomic Adsorption Spectrophotometric (AAS) method.

3.2.3 Survival rate of yeast strains in GYP medium supplemented with Mn(II) concentrations under stress of ^{60}Co-gamma irradiation, hydrogen peroxide and menadione

After the pre-cultured overnight with shaking 300 rpm, at 30^{o}C, the initial volume of 1/100 suspension of the pre-culture suspension was grown in 10 ml GYP media supplemented with various manganese ion concentrations of 0 mM (control sample), 0.5 mM, and 1.0 mM. Then yeast cells were collected by centrifugation 4000 rpm, 5 min at normal temperature by TOMY high speed micro refrigenated centrifuge MRX 150 and Himae CR 21 with rotor R21A-26, 46. Yeast cells after diluted by saline solution approximately 10^7cells/ml of 10ml solution were put into a test tube with topper and irradiated with gamma irradiation doses of 0 kGy, 0.5 kGy, 1.0 kGy, and 2.0 kGy.

With the stress of hydrogen peroxide, after cultured in GYP media supplemented with various Mn(II) concentrations, yeast cells were collected and washed twice in saline by centrifugation 4000 rpm, 5 min at normal temperature. After diluted by fresh GYP medium, yeast cells were directly stressed by H_2O_2 concentration of 3.0 mM for

1h under shaking 120 rpm, 30°C.

With the stress of menadione, after cultured in GYP media supplemented with various Mn(II) concentrations, yeast cells were collected and washed twice in saline by centrifugation 6000 rpm, 5 min at normal temperature. After diluted by fresh GYP medium, yeast cells were directly stressed by menadione concentration of 0.5 mM for 30 min with shaking 120 rpm, 30°C.

After stressed with ROS sources, cell suspensions were diluted by saline solution and spread out in GYP-agar plates and incubated 3 – 4 days for colonies growing. Viable cell numbers were determined by counting the colonies that formed on GYP agar plates after stressful conditions at 30°C. Sensitivities were determined as survival percentage values, which were calculated by dividing the viable cells numbers after stress application by the viable cells numbers determined under the same growth conditions but without the stress exposure.

3.2.4 Detection of ROS production using DCFH-DA

After pre-cultured, 1/100 volume of overnight culture was cultured in 10 ml fresh GYP media that supplemented with manganese concentration of 0 mM, 0.25 mM, 0.5, and 1.0 mM for 24 h with shaking 120 rpm, 30°C. Then cell pellets were collected and washed by centrifugation 4000 rpm, 5 min, 4°C. Cell pellets were re-suspended in 0.2 M potassium phosphate buffer pH 6.0. From 5mM H_2DCFDA dissolved in ethanol, add to the 10ml culture to a final concentration of 20 μM. After incubated at 30°C for 15 min, cell pellets were collected and washed twice with phosphate buffer pH 6.0 by centrifugation 4000 rpm, at 4°C, 5 min. Cell lysed by adding 0.5 ml phosphate buffer

and plus 1.5g glass beads 0.4 mmϕ, agitation on a Voltex mixer 1 min, then, keep on ice 1 minute (7 cycles). Supernatant was collected by centrifugation 15000 rpm at 4ºC, 10 min and analyzed by Fluorescent spectrophotometry set an excitation of 490 nm and an emission wavelength band between 500 and 600 nm. All fluorescence measurements were performed at room temperature.

3.2.5 Catalase Activity

Yeast cells after pre-cultured in 5 ml GYP medium overnight with shaking 300 rpm, at 30ºC, the volume of initial cells 1/100 was transferred to new 10 ml GYP media that supplemented with 0 mM, 0.5 mM, and 1.0 mM manganese (II) ion concentration and incubated with shaking 120 rpm, 30ºC for 24h. Yeast cells were harvested and re-suspended in 0.2 M potassium phosphate buffer (pH 7.4) to obtain an absorbance of 0.1 at a wavelength of 600 nm. Cells were lysed using a solution containing 0.5 mm diameter glass beads in microfuge tubes using a Bioraptar ultrasonic fragmentation device. Mixtures of yeast cells and glass beads were inserted into the apparatus and sonicated for 7 minutes at 30 s intervals, and the cooling condition was performed by a cooling pump. Samples were centrifuged at 15000 x g for 10 minutes, after that the supernatants were collected. Catalase activity was measured by the breakdown of hydrogen peroxide, and this was followed directly by a decrease in absorbance at 240 nm. The protein contents of the enzyme solutions were measured by the Bradford Assay method. OD values were recorded at 595 nm.

3.2.6 Trehalose content

Trehalose concentration was analyzed following the assay procedure of Megazyme KIT K-treh 06/11. After cultured with the same process above, yeast cells of 10 ml suspension were collected by centrifugation 4000 rpm, 5 min, 4^{o}C. Cell pellets were transferred to Eppendorf tubes and washed with 1ml saline solution twice by centrifugation 6000 rpm, 5 min, 4^{o}C. After adding 0.3 ml distilled water, the tubes were placed on a magnetic stirrer and stir for 15 minutes at 100^{o}C until the sample is completely dispersed. Cooling to room temperature before centrifugation 6000 rpm, 5 min, 4^{o}C to keep supernatant. The volume of 0.2 ml sample solution was used to analyze trehalose following the KIT procedure.

3.2.7 Supperoxide dismustase activity

SOD activity was analyzed following the assay procedure of SOD Assay Kit-WST. After pre-cultured, 1/100 volume of overnight culture was cultured in 10 ml fresh GYP media that supplemented with manganese concentration of 0.5, 1.0, 2.0 mM for 24h with shaking 120 rpm, 30^{o}C. Then cell pellets were collected by centrifugation 4000 rpm, 5 min, 4^{o}C. Cell pellets were re-suspended in 0.2 M potassium phosphate buffer pH 7.4. Cells were lysed using a solution containing 0.5 mm diameter glass beads in microfuge tubes using a Bioraptar ultrasonic fragmentation device. Mixtures of yeast cells and glass beads were inserted into the apparatus and sonicated for 7 minutes at 30 s intervals, and the cooling condition was performed by a cooling pump. Samples were centrifuged at 15000 x g for 10 minutes, after that the supernatants were collected. Total SOD activity of crude enzymes was analyzed following the process of SOD Assay Kit-

WST. *"One unit of SOD is defined as the amount of the enzyme in 20 µl of sample solution that inhibits the reduction reaction of WST-1 with superoxide anion by 50%"*.

3.3 RESULTS AND DISCUSSIONS

3.3.1 Screening of the growth profile of Saccharomyces cerevisiae BY4741 and Saccharomyces cerevisiae IM3 in media supplemented with various manganese concentrations

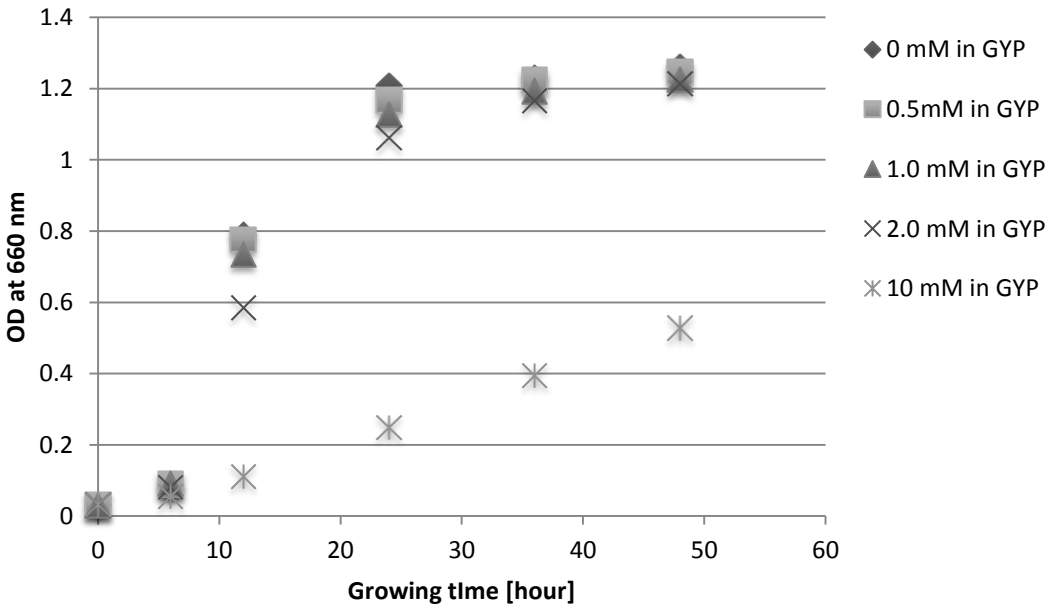

Fig. 3.1. The growth profiles of *S. cerevisiae* BY4741 in GYP media supplemented with manganese (II) ion concentrations of 0 mM, 0.5 mM, 1.0 mM, 2.0 mM, and 10 mM.

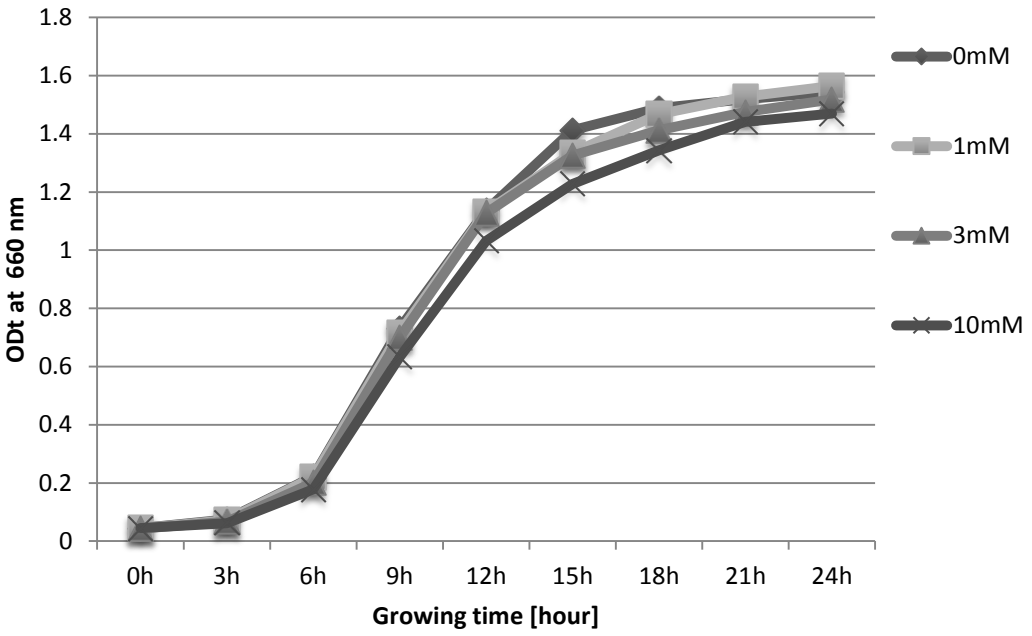

Fig. 3.2. The growth of *S. cerevisiae* IM3 in media supplemented manganese ion concentration of 0 mM, 1.0 mM, 3.0 mM, and 10.0 mM.

We can easily to see from Figure. 3.1 that Mn concentration supplemented into GYP media at low concentrations (0.5 mM, 1.0 mM) were not affected on to the growth of *S. cerevisiae* BY4741. Although at 2.0 mM Mn-supplemented into nutrient the growth of yeast showed little lower than the lower concentration before 24 hours cultivated, yeast cells were still growing at the same rate to get the stationary phase after 24 hours. At the highest Mn concentration supplemented 10 mM, Mn(II) ion became toxic to the yeast cells. The growth curve of *S. cerevisiae* BY4741 at this condition was inhibited and OD values were very low compared with others. This trend quite different with *S. cerevisiae* IM3 strain, at 10mM Mn concentration, the growth of *S. cerevisiae* IM3 was not affected. These results suggest that *S. cerevisiae* IM3 has a mechanism that induces more tolerant to the cytotoxicity of Mn(II) ion than *S. cerevisiae* BY4741.

When both yeast strains were cultured in high Mn condition, the Mn accumulation in yeast cells was recognized. The manganese accumulation in IM3 was four to five fold more than in BY4741 (Table 3.1).

TABLE 3.1. Manganese accumulation of yeast cells after 24h cultivated in GYP media supplemented with various Mn(II) ion concentration

Strain	Mn(II) ion concentration (mM)			
	0*	5*	10*	15*
BY4741	0	597	1380	2060
IM3	0	2390	6850	8010

*Supplemented Mn(II) ion concentration in medium

These results suggested that more tolerance to the cytotoxicity of Mn(II) ion in *S. cerevisiae* IM3 than in *S. cerevisiae* BY4741 due to the more accumulation of manganese in *S. cerevisiae* IM3 cells than in *S. cerevisiae* BY4741 cells.

3.3.2 Survival rate of yeast strains under stress of hydrogen peroxide and menadione

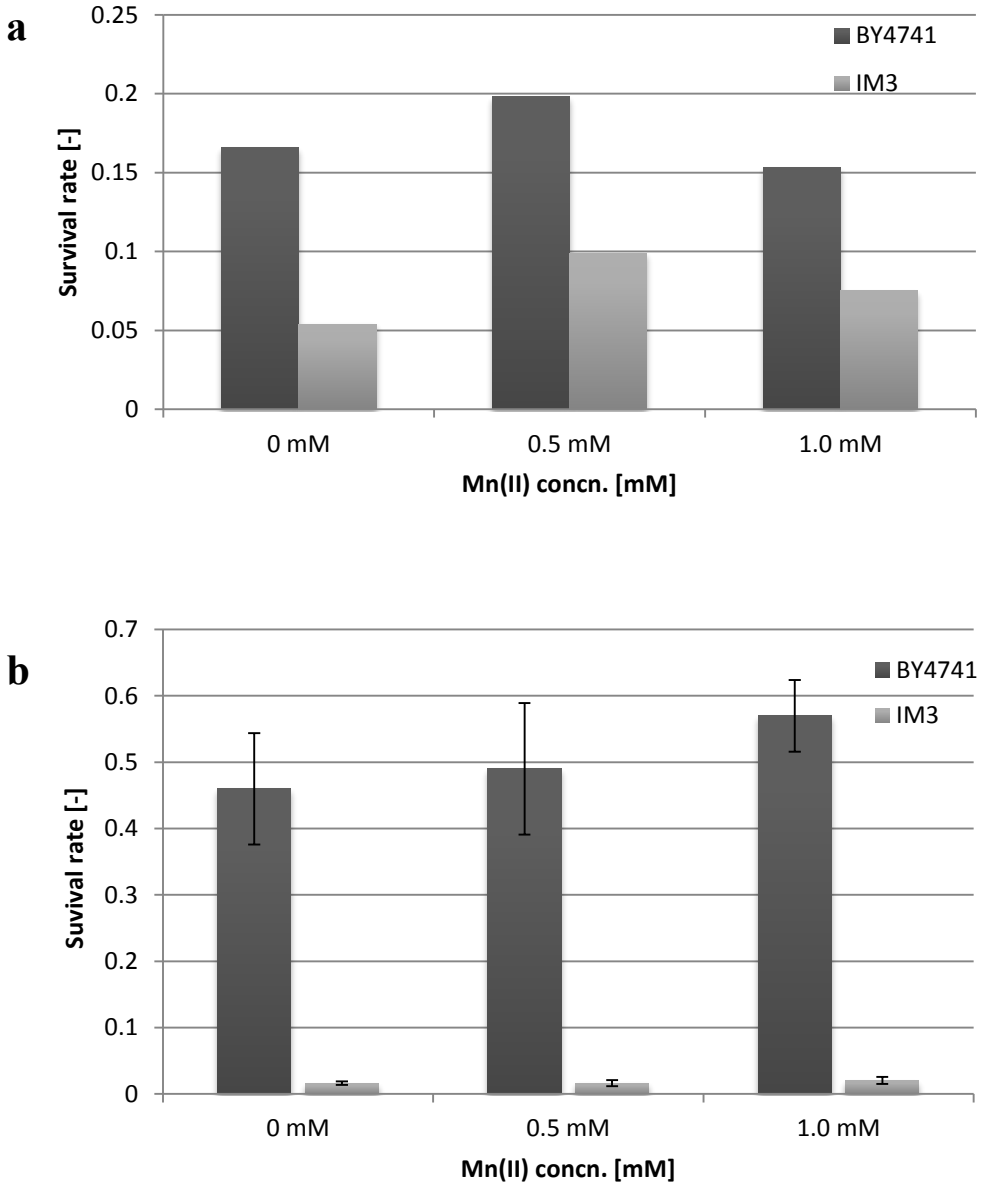

Fig. 3.3. Survival rate of yeast cells of *S. cerevisiae* BY4741 and *S. cerevisiae* IM3 cultured in GYP media supplemented with 0 mM, 0.5 mM, and 1.0 mM Mn(II) ion concentration. (3a.) under stress of 0.5 mM menadione; (3b.) under stress of 3 mM hydrogen peroxide.

Oxidative stress generated by H_2O_2 frequently induces oxidative damages in biomolecules such as proteins, lipid, and DNA. However, a reduction in lipid and protein oxidation is observed in cells pre-adapted and subsequently exposed to H_2O_2 (Benaroudi et al., 2001; Nery et al., 2008). The role of Mn as an antioxidant is likely to involve redox reactions between the Mn(II) and Mn(III) states.

In the results from figure 3.3a. and 3.3b., even under stress of 3 mM hydrogen peroxide the function of Mn(II) to protect yeast cells was not significantly shown by the survival rate of yeast cells when Mn(II) ion supplemented in GYP media, the survival rates at 0.5 mM and 1.0 mM Mn(II) ion concentration showed slightly higher than without Mn(II) supplemented.

In addition, under the stress of 0.5 mM menadione, when 0.5 mM Mn(II) supplemented in media, yeast cells were got higher survivable ability than 1.0 mM Mn(II) supplemented and also without Mn(II) supplementation in both strains.

However, under the stress sources, such as hydrogen peroxide and menadione, yeast cells of *S. cerevisiae* IM3 strain showed very low survival rate compared with its parental strain, especially under the stress of hydrogen peroxide, the survival rates of *S. cerevisiae* IM3 strain was lower at least 25 fold compared with *S. cerevisia* BY4741. In the case of stressed by menadione, the survival rate of *S. cerevisiae* IM3 was lower over 2 fold compared to its parental strain. It is also suggested for the toxicity of hydrogen peroxide higher than menadione.

It is suggested that whether the activity of enzymes, such as catalase, superoxide dismutase plays a key role to protect cells or Mn ion function in cells also act independently with enzymes?

3.3.3 Manganese supplemented in nutrient helps yeast cells more resistant to oxidative stress caused by gamma-radiation doses

Are there any effects of gamma-radiation doses to the resistance of yeast? Yeast cells at manganese concentrations that have shown no effect to the growth curves were used to exposed to gamma-radiation doses of 0 kGy, 0.5kGy, 1.0 kGy, and 2 kGy to check the effect of gamma-irradiation doses.

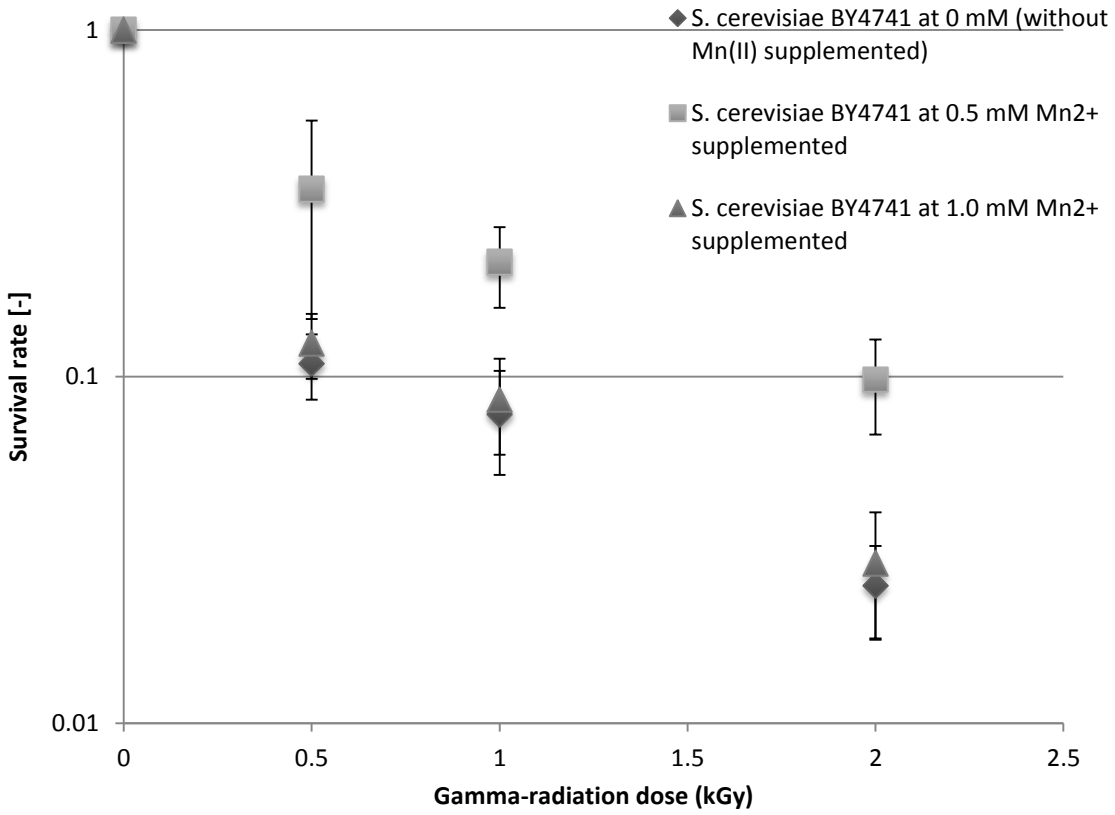

Fig. 3.4. Survival rates of *S. cerevisiae* BY4741 that were grown in GYP media supplemented with manganese concentration of 0 mM, 0.5 mM, and 1.0 mM exposed to gamma-irradiation doses of 0 kGy, 0.5 kGy, 1.0 kGy, and 2.0 kGy

As we can see from Figure 3.4. above, at all gamma-irradiation doses, yeast cells that collected from 0.5 mM manganese supplemented in nutrient show higher resistant above all others. Although yeast cells at 1 mM manganese supplemented have the resistance with gamma-irradiation miniature higher than yeast cells collected from normal GYP media, the survival rates of yeast at this condition were plentiful lower than yeast cells at 0.5 mM manganese ion supplemented in the nutrient.

It can be assumed that manganese ion concentration about 5 – 7 nmol/10^9cells has the highest gamma-irradiation resistance (Table 3.2). Intracellular manganese concentrations in which have no effects on the growth of yeast can help yeast cells more resistant to gamma-irradiation than without or less manganese ion in yeast cells.

TABLE 3.2. Mn concentration of yeast cells in GYP medium supplemented with 0.5, 1.0 mM Mn(II) before and after irradiation

Strain	Mn(II) concn. supplemented in GYP (mM)	Mn(II) concn. before irradiation (nmol/109cells)	Mn(II) concn. after irradiation (nmol/109cells)
BY4741	0	-	-
	0.5	5.01 ± 0.64	-
	1.0	76.14 ± 5.49	6.83 ± 1.61
IM3	0	-	-
	0.5	59.91 ± 3.66	3.03 ± 0.67
	1.0	189.22 ± 10.32	24.65 ± 5.38

(-): none detected by AAS

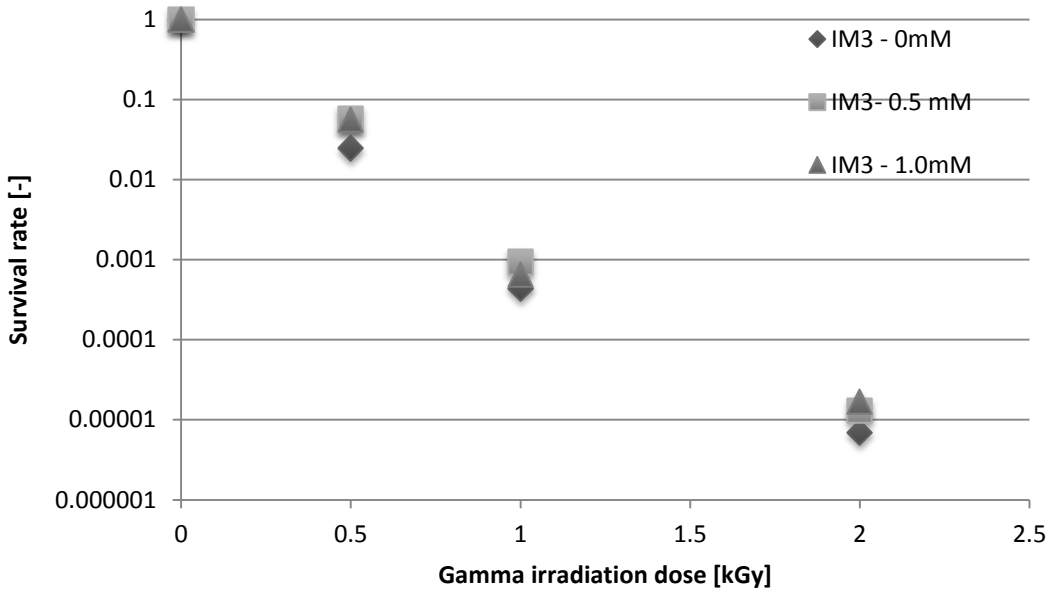

Fig. 3.5. Survival rates of *S. cerevisiae* IM3 that were grown in GYP media supplemented with manganese concentration of 0 mM, 0.5 mM, 1.0 mM, exposed to gamma irradiation doses of 0 kGy, 0.5 kGy, 1.0 kGy, and 2.0 kGy.

Although, manganese concentration supplemented in media also helped yeast cells of *S. cerevisiae* IM3 strain a little bit more resistant to gamma irradiation doses, compared with *S. cerevisiae* BY4741 this strain is very sensitive to gamma irradiation.

The question must be answered that if followed the theory of some references, the higher level of manganese ion should help cells higher resistant to gamma irradiation doses, so that, what makes different of this strain? Are there any possibility that this is a mutant strain or what are factors involved with gamma irradiation resistant than manganese ion function?

The checking of Catalase activity and also Trehalose expression may be can

answer the role of manganese ion get more function superoxide scavenging ability.

3.3.4 Detection of ROS production using DCFH-DA

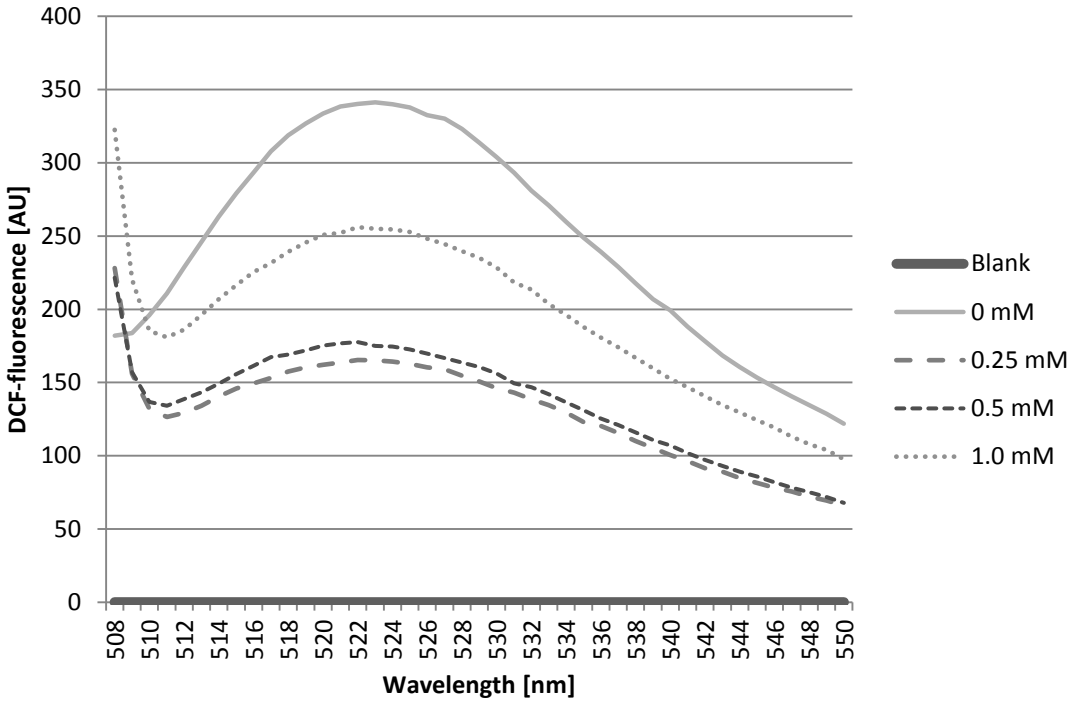

Fig. 3.6. DCF fluorescence intensities from *S. cerrevisiae* BY4741 cells at 24 h cultivated in GYP media supplemented with Mn(II) ion concentrations of 0 mM, 0.25 mM, 0.5 mM, and 1.0 mM.

Intracellular oxidation is one of the best characterized and explored biomarkers use to detect oxidative stress. In this work, using the fluorescent probe 2',7'-Dichlorofluorescein diacetate (H2DCF-DA) to determine the levels of intracellular oxidation during H_2O_2 stress. H2DCF-DA is very sensitive to several ROS and can be oxidized to a highly fluorescent 2',7'-dichlorofluorescein (DCF) (Rastogi et al., 2010).

Oxidative stress generated by H_2O_2 frequently induces oxidative damages in biomolecules such as lipid, proteins and DNA (Benaroudj et al., 2001; Nery et al., 2008).

In this result, we can easily to see the trend that when manganese supplemented in nutrient lower 0.5 mM, the DCF-fluorescence of these concentration conditions showed significantly lower than higher concentration from over 0.5 mM such as 1.0 mM. Especially, at 1.0 mM, DCF-fluorescence of yeast showed very high. It means that intracellular oxidation of yeast cells in this condition shows the oxidative stress is high. This result was also argued to the growth curve of yeast cells at 1.0 mM Mn, in which Mn becomes toxic to the growth of yeast cells, showed slightly lower than yeast cells at normal medium

This result maybe can suggest for the result that yeast cells at lower 0.5 mM manganese can more resistant to reactive oxygen species than at higher concentrations. In other words, manganese ion may function as an ROS scavenger.

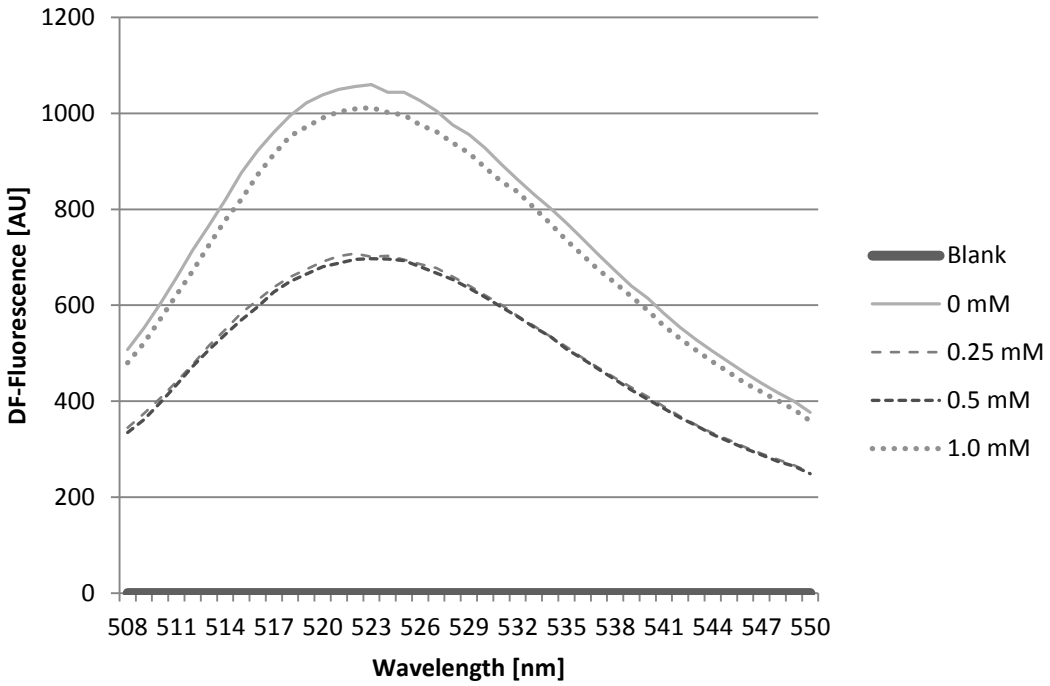

Fig. 3.7. DCF fluorescence intensities from *S. cerrevisiae* IM3 cells at 24 h cultivated in GYP media supplemented with Mn(II) ion concentrations of 0 mM, 0.25 mM, 0.5 mM, and 1.0 mM.

The same trend occurs with *S. cerevisiae* IM3. The DCF-fluorescence of these concentration conditions showed significantly lower than the higher concentration of 1.0 mM, in this condition, DCF-fluorescence of yeast showed very high. It means that intracellular oxidation of yeast cells in this condition shows the oxidative stress is high. Even the behavior of intracellular oxidation of *S. cerevisiae* IM3 shows the same trend with *S. cerevisiae* BY4741, the DCF-fluorescence of yeast cells at all concentration of Mn shows three to over five-fold compared with *S. cerevisiae* BY4741, it is also suggesting for the reason why *S. cerevisiae* IM3 more sensitive with ROS than the parental strain.

3.3.5 Catalase activity of Saccharomyces cerevisiae BY4741 and Saccharomyces cerevisiae IM3 in nutrient supplemented with Mn(II) ions

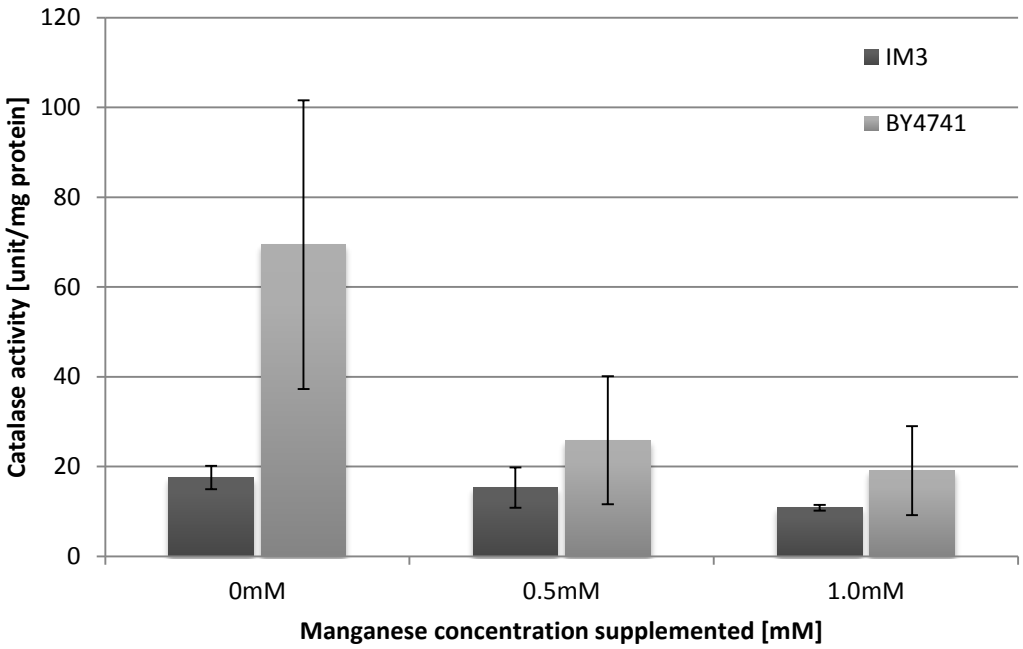

Fig. 3.8. Catalase activity of *S. cerevisiae* BY4741 and *S. cerevisiae* IM3 in GYP media that supplemented with 0.5 mM and 1.0 mM manganese.

Manganese may act as an antioxidant by functionally substituting for some Fe enzymes (catalase is one of the group), thereby mitigating the potential for deleterious Fenton reactions at enzyme active sites (Anjem et al., 2009; Sobota and Imlay, 2011). Regardless of the mechanism, it was unclear whether the protective effect of Mn is part of the cell's tightly regulated battery of antioxidant responses or is a passive unregulated process. Reddi and Culotta (2011) has been provided an evidence when *S. cerevisiae* was used that Mn antioxidant is a component of the oxidative stress defense that is regulated through nutrient-sensing pathways. Furthermore, Nishimoto et al. (2014) have

been reported about catalase activity to help *S. cerevisiae* more tolerant to gamma irradiation.

It was previously proposed that formation of manganese antioxidants may be constitutive, requiring little energy input from the cell and that only the enzymes that remove reactive oxygen (e.g. SOD, peroxidases, and catalases) are regulated during oxidative stress (Horburgh et al., 2002). The result of Aguirre and Culotta (2012) was provided evidence to the contrary that likes their enzymatic counterparts, the formation of non-proteinaceous manganese antioxidants is tightly regulated according to cellular need.

In our work, even though manganese ion supplemented in nutrient caused catalase activity lower and lower when manganese ion supplemented more, yeast resistance with gamma-radiation was higher than the normal condition (Fig. 3.8). It is suggested that catalase activity is not the primary factor for oxidative scavenge in yeast cells when manganese ion becomes competitive with the iron of Catalases.

There are various evidences of reference proved manganese function as an antioxidant but its functions as intracellular physiology antioxidant still in conflict theories.

3.3.6 Trehalose concentrations in yeast cells at nutrient conditions that supplemented with Mn(II) ion concentrations

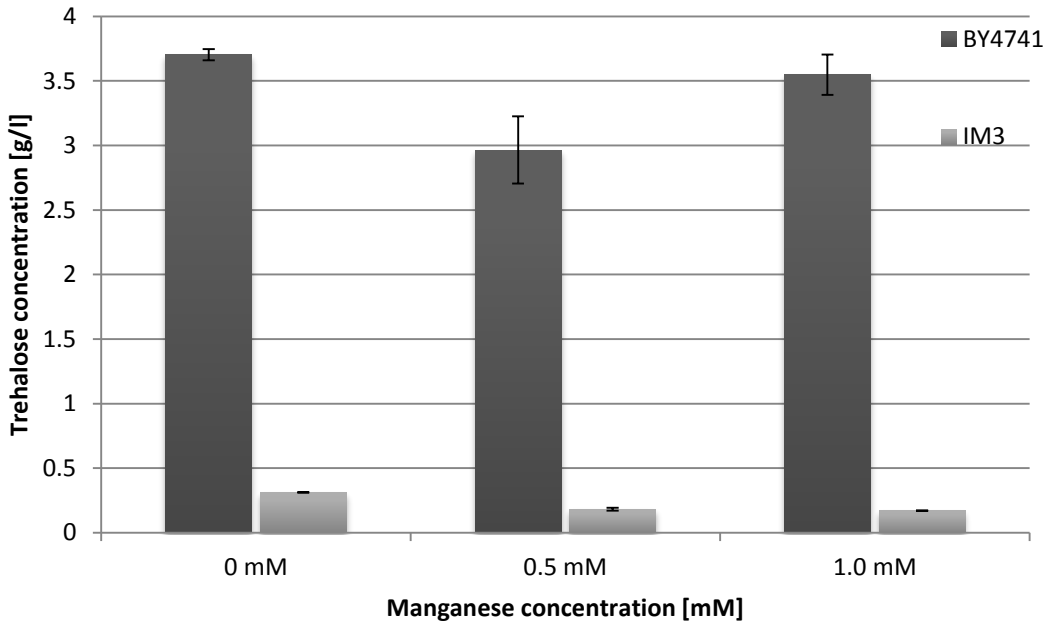

Fig. 3.9. Trehalose concentration of yeast cells in nutrient supplemented with manganese ion concentration of 0 mM, 0.5 mM, and 1.0 mM

Following Figure 3.9., concentrations of Trehalose at all conditions of *S. cerevisiae* BY4741 were not so different. Maybe at these conditions, manganese concentrations do not become toxic to yeast cells. So that the expression of trehalose in yeast was not getting high when manganese ion supplemented high to 1.0 mM.

Even at 0.5 mM Mn condition, trehalose concentration is a little lower than at 1.0 mM Mn, and normal GYP, yeast cells are more tolerant to gamma-irradiation. In this case, it means that trehalose has not got the function to protect yeast cells to gamma-irradiation than manganese ion.

For *S. cerevisiae* IM3, trehalose concentrations were a little bit lower when higher manganese ion supplemented in media.

Compared with *S. cerevisiae* BY4741, the trehalose concentrations of IM3 were less 10-fold. Maybe trehalose function to help BY4741 more tolerant with gamma irradiation doses than IM3, but, it is not significantly can be prove the trehalose function as superoxide scavenging ability in case of IM3.

3.3.7 Superoxide dismutase activity of S. cerevisiae BY4741 in nutrient supplemented with Mn(II) ions

Several enzymes such as superoxide dismutase (SODs), catalase (CAT) and peroxidase (POD) are antioxidant enzymes that act as a major function against reactive oxygen species (ROS) which are causing oxidative damage and may possibly result in cell death.

For *S. cerevisiae* BY4741 strain, SODs activities of yeast cells at control sample, 1.0 mM and 2.0 mM manganese supplemented were showed the same level. Interestingly, SOD activity at 0.5 mM manganese supplemented in nutrient was highest compared with others concentration of manganese supplemented in GYP media. Also, at this condition yeast cells were more resistant to gamma-irradiation doses than the others. Thus, manganese concentrations supplemented into nutrient in which has no effect on the growth of yeast help yeast cells express more SOD enzymes and help yeast cells more tolerant with gamma-radiation doses than the normal cells or yeast cells cultured in nutrient conditions supplemented manganese concentrations that become toxic to yeast cells.

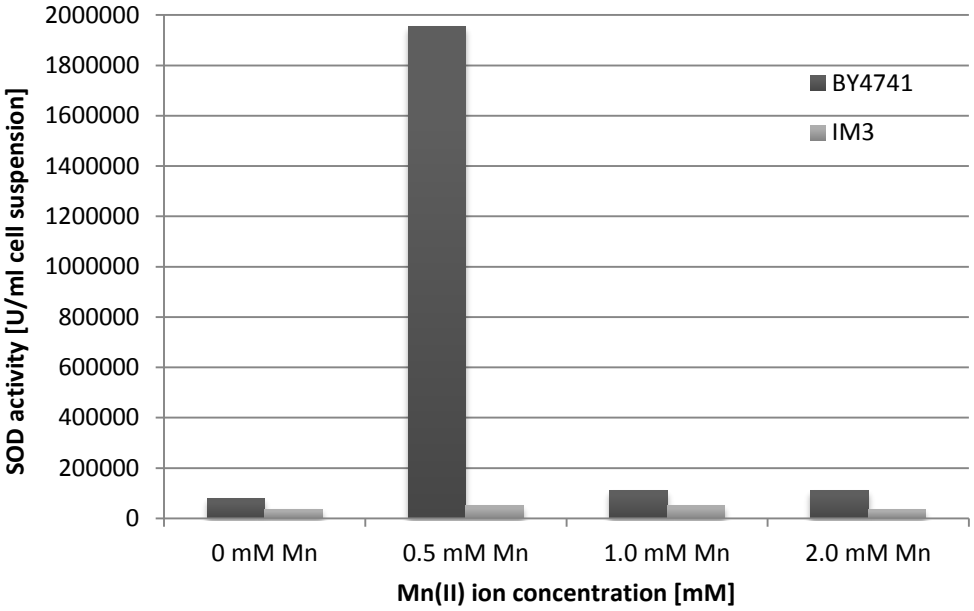

Fig. 3.10. SOD activity of 1ml cell suspension of *S. cerevisiae* BY4741 and *S. cerevisiae* IM3 in GYP media supplemented with 0, 0.5, 1.0 and 2.0 mM Mn(II) ion concentration.

For *S. cerevisiae* IM3, SODs activities of yeast cells at all Mn(II) ion concentrations supplemented in GYP media showed almost the same level. It was also explained for the reason why S. cerevisiae IM3 are very sensitive with not only ROS caused by ^{60}Co-gamma irradiation but also other ROS sources, such as H_2O_2 and menadione.

3.4 CONCLUSION

We found that manganese did not appear to be toxic as supplementation did not negatively affect the development of yeasts. In addition, the oxidatively challenged of *S. cerevisiae* BY4741 strain was significantly increased after Mn supplementation.

Manganese ion concentration about 5 – 7 nmol/10^9cells has the highest gamma-irradiation resistance. Intracellular manganese concentrations in which have no effects on the growth of yeast can help yeast cells more resistant to gamma-radiation than without or less manganese ion in yeast cells.

The yeast strain – *S. cerevisiae* BY4741 showed slightly resistant to gamma-irradiation when manganese ion was supplemented in growth media, especially at the concentration of 0.5 mM, which has no adverse effect on the growth of yeast, suggesting that cellular manganese might be contributing for preventing oxidative stress caused by gamma- irradiation. At this concentration of Mn(II), the activity of SODs showed significantly higher than without Mn(II) supplementation and higher Mn(II) concentration supplemented in media, suggested the necessary concentration level of Mn for protecting yeast cells to ROS caused by various sources, such as gamma-irradiation, menadione, and hydrogen peroxide.

The active oxygen scavenging enzymes catalase did not show activity enhance even were lower when higher Mn(II) concentration supplemented in media, and also trehalose contents were similar at all manganese concentrations supplemented in the media, suggested that manganese ion might protect the yeast cells independently with catalase and trehalose contents in yeast cells.

Manganese(II) ion supplemented in medium enhance the expression of total

superoxide dismutase at concentration 0.5 mM or lower, suggesting that Mn-SOD plays an important role in protecting yeast cells against gamma-irradiation caused by ^{60}Co-gamma rays.

S. cerevisiae IM3, the mutant accumulating manganese in its cell, showed more sensitive to not only gamma-irradiation but also ROS caused by hydrogen peroxide and menadione than the parent strain BY4741, precultured under Mn(II) supplemented or Mn free condition. These results may show that intracellular Mn contributes the response to the damages from gamma-irradiation, especially response to the oxidative stress derived from the indirect action caused by gamma-irradiation and also ROS caused by other sources. When Mn-supplemented in medium, the catalase activity was gradually lower in *S. cerevisiae* BY4741. However, the catalase activity of IM3 was about 1/3 than that of BY4741 in Mn-free culture and was little changed in addition of Mn(II). These results suggested that the catalase production is deficient in IM3 under Mn-rich culture. The relationship for Mn(II) ion and the oxidative stress scavenging enzymes in yeast cells is interesting and can be analyzed in next study. Although Mn(II) appears to be beneficial to yeasts for protecting yeast cells to ROS, the mode of action remains unclear. Manganese may work directly as a free radical scavenger, as it has been postulated to do so in unicellular organisms, or may work indirectly by up regulating several protective factors.

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CHAPTER 4

SUMMARY AND FUTURE PERSPECTIVES

4.1 SUMMARY

S. cerevisiae, that has many advantages such as easy to cultivate at large-scale, high-biomass, easy to manipulate, generally recognized as safe microorganisms, especially can accumulate a large range of metals, is a possible alternate promising tool in the metal removal of water containing heavy metals, and establishes a new opportunity for the taking benefit from yeast biomass. The information reviewed above indicate that bioremediation using yeast cells of *S. cerevisiae* is a novel method to pure of metal contaminant from real water or wastewater containing heavy metals. It is evident that the bioremediation of yeast cells is not only realized in passive uptake, which in sort biosorption processes are thought to be physical adsorption or ion exchange at the cell surface, but also active uptake which also metal ions takes place slowly and mainly based on metabolism processes of living cells. The combination of these processes together with other non-biotechnology-based processes may also helpful for enhancing the efficiency of a real practical system in large-scale, for simultaneous removal of organic substances and heavy metal ions from aqueous solution.

To an extent for application of yeast cells, the isolation of super-resistant accumulation strains from contaminated sites has to consider. Genetic engineering may further enhance the potential of vigorous environmental strains, but till now the existence of legal and sociopolitical barriers does not permit engineered yeast cells to release into the environment. Moreover, even some of reactor designs have been successful at pilot scale for the treatment of real effluents, a range of reactors should be proposed.

Due to the flocculent properties of the yeast cell strains, we can take advantages

of the auto-aggregation properties of these yeast cells, which over come the need for cell immobilization or the use of solid-liquid separation technique, and in some case, equipment of treatment plants, such as sedimentation tanks, can be used for this purpose with an economical point. Together, these are important characteristics of a low-cost and competitive technology when the advantages of reactors are simple to design, operational stability and low operational and maintenance costs.

The present doctoral thesis focused on setting for the results obtained in the studies of bioaccumulation of Mn from solution through growing cells to evaluate Mn-accumulation or Mn-absorption capacity of *S. cerevisiae*. Most of the studies dealing with microbial metal remediation via growing cells describe the biphasic uptake of metals, i.e., the initial rapid phase of biosorption followed by slower, metabolism-dependent active uptake of metals (Donmez and Aksu, 1999; Garnham et al., 1992). Nevertheless, there are significant practical limitations to bio-uptake by living cell systems such as sensitivity of the system to extremes of pH, high metal/salt concentration and requirement of external metabolic energy (Donmez and Aksu, 2001). Finding new resistant and higher accumulation strain that has better ability to absorb or accumulate Mn, and optimize the growth of Mn resistant strain using some parameters such as temperature, pH, and the Mn absorption of the mutant strain.

This study revealed that Mn accumulation/adherence ability of *S. cerevisiae* BY474 strain was believed appropriate in some studies, the Mn accumulation/adherence concentration of yeast cells was still only at a mediocre level (Kratochvil and Volesky, 1998). In addition, the Mn absorption capacity of yeast cells may be dependent on the activities of the cells, or on adherence to the cell surface by the function groups in the cell wall. Our results indicated that Mn accumulates most during the exponential growth

phase, suggesting that the Mn content of yeast cells is dependent on cell activities. Mn-absorption ability of *S. cerevisiae* BY4741 and the variant tolerant strain *S. cerevisiae* IM3 closely related to activities of yeast cells during their life cycle. Without nutrients and without activity (i.e., dead cells) yeast does not absorb Mn. Living cells can absorb over hundred- to thousand-fold the Mn as compared to non-living cells. Based on the data obtained, these results showed that formation of *S. cerevisiae* IM3 in the YPD medium without Mn supplementation was the same as the parental strain BY4741, but in Mn supplementation conditions, *S. cerevisiae* IM3 exhibited four- to five-fold the Mn accumulation than the parental strain. This suggests that this strain could be a good candidate for practical application using living cells. Another should be practiced way of producing more resistant and efficient strain is through adaptation of the cells to progressively higher concentrations of Mn which expected that adapted cells can have more metal uptake capacity and grow well in the presence of higher Mn concentration than non-adapted cells to avoid the legal and socio-political barriers of the using genetically engineered yeast cells.

The role of variety powerful enzymes, such as catalase, peroxidase, superoxide dismutase in subtracting or invalidating ROS is well-documented; however, in aerobic organisms, not only enzymatic systems but also non-enzymatic factors that collectively detoxifying reactive oxygen species can play an important role. Not much is known in the class of non-enzymatic factors for protection against ROS in a variety of organisms, especially in yeasts or other eukaryotic cells. Mn(II) and/or Mn-complexes antioxidants are well known for their role in displacing SOD functions to protect cells of *Deinococcus*, *Lactobacillus plantarum,* and *Escherichia coli*, but less understood in the class of *S. cerevisiae*.

In the framework of this thesis, intracellular Mn(II) ion concentration about 5 – 7 nmol/10^9cells of *S. cerevisiae* BY4741 has the highest gamma-radiation resistance. Mn concentrations in which have no effects on the growth of yeast can help yeast cells more resistant to ROS caused by gamma-radiation, hydrogen peroxide, and menadione than without or less manganese ion in yeast cells.

The yeast strain – *S. cerevisiae* BY4741 showed slightly resistant to gamma-irradiation when manganese ion was supplemented in growth media, especially at the concentration of 0.5 mM, which has no adverse effect on the growth of yeast, suggesting that cellular manganese might be contributing for preventing oxidative stress caused by gamma-irradiation. At this concentration of Mn(II), the activity of SODs showed significantly higher than without Mn(II) supplementation and higher Mn(II) concentration supplemented in media, suggested the necessary concentration level of Mn for protecting yeast cells to ROS caused by various sources, such as gamma-irradiation, menadione, and hydrogen peroxide.

The active oxygen scavenging enzymes catalase did not show activity enhance even were lower when higher Mn(II) concentration supplemented in media, and also trehalose contents were similar at all manganese concentrations supplemented in the media, suggested that manganese ion might protect the yeast cells independently with catalase and trehalose contents in yeast cells.

Manganese(II) ion supplemented in medium enhance the expression of total superoxide dismutase at concentration 0.5 mM or lower, suggesting that Mn-SOD plays an important role in protecting yeast cells against gamma-irradiation caused by ^{60}Co-gamma rays.

S. cerevisiae IM3, the mutant accumulating manganese in its cell, showed more sensitive to not only gamma-irradiation but also ROS caused by hydrogen peroxide and menadione than the parent strain BY4741, precultured under Mn(II) supplemented or Mn-free condition. These results may show that intracellular Mn contributes the response to the damages from gamma-irradiation, especially response to the oxidative stress derived from the indirect action caused by gamma-irradiation and also ROS caused by other sources. When Mn-supplemented in medium, the catalase activity was gradually lower in *S. cerevisiae* BY4741. However, the catalase activity of IM3 was about 1/3 than that of BY4741 in Mn-free culture and was little changed in addition of Mn(II). These results suggested that the catalase production is deficient in IM3 under Mn-rich culture. The relationship for Mn(II) ion and the oxidative stress scavenging enzymes in yeast cells is interesting and can be analyzed in next study. Although Mn(II) appears to be beneficial to yeasts for protecting yeast cells to ROS, the mode of action remains unclear. Manganese may work directly as a free radical scavenger, as it has been postulated to do so in unicellular organisms, or may work indirectly by upregulating several protective factors.

4.2 FUTURE PERSPECTIVES

S. cerevisiae, a promising tool for metal removal because of its advantages and unique characteristics, has received increasing attention. Bioaccumulation/biosorption using yeast cells is basically at lab scale in spite of its development during the past decades. Even, successful commercial biosorbents were reported mainly in different types of biomaterial such as bacteria, algae, fungi, etc. namely AlgaSORB^{TM} (C.

vulgaris), AMT-BIOCLAIMTM (Bacillus biomass), BIO-FIX (peat moss, algae, bacteria, yeast), (Wang and Chen, 2009) the using of yeast cells should be considered.

The mechanisms involved in either bio-adsorption or bioabsorption/bioaccumulation should be further studied with more efforts by utilizing various model techniques and the combination of them (Kratochvil and Volesky, 1998). The mechanisms of metal accumulation and also important influencing factors, such as pH, competitive ions or co-ions, have not been fully understood. The optimization of the parameters of biosorption/bioaccumulation processes, including reuse and recycling, such as physicochemical conditions, fluid dynamics, engineering reactors, should be studied more in details.

To screen and select the most promising yeast strains with high metal accumulation capacity and selectivity for heavy metal ions are required for a full-scale bioaccumulation/biosorption process, it is suggested the first major important factor in the bioaccumulation/biosorption using yeast cells.

Based on these facts, the development of systems using microbial biomass as a continuous system to remove metals based only on bio-adsorptive/adsorption appears to be impossible or uneconomic. However, application of growing cells and active cells for continuous systems not only at the laboratory-scale, but also for real treatment systems, might be a better candidate because of the abilities of living cells, such as continuous metabolic absorption of metals and self-replenishment. Furthermore, the metals that penetrated into the cells were detoxified during metabolic activities of cells that combined them to intracellular proteins or chelatins before being integrated into vacuoles and other intracellular organelles. This results in detoxified metals and often reduces the risk of metals returning to the environment (Gekeler *et al.,* 1998). In

addition, use of growing cultures may allow for the exploitation of the process for the removal of many kinds of pollutants. Living cells have almost unlimited capacities to degrade organic materials, even organic metallic complexes, and uptake inorganic ions such as ammonium, nitrate, and phosphate. Although several studies on the application of living cells for treatment of metal ions have been conducted, the practical application of growing cells still face factors, such as extreme pH, high salt concentration, and energy requirements (Donmez and Aksu, 2001). Thus, finding suitable methods and microbial strains of growing cells that can maintain their accumulation capacity after multiple bio-absorption-desorption cycles, have high metal bioaccumulation, and grow well under environmental stress is required for practical application.

Non-enzymatic factors, especially “Mn-antioxidant”, are powerful shields for microorganisms against the ionizing effects of radiation and other oxidative damage. There is a need to increasing evidence in all branches of life. Thus far, Mn(II) functions in antioxidant defense and its behavior in detoxification should be studied and identified. *S. cerevisiae* IM3, the mutant have a significant Mn accumulation, showed more sensitive to not only gamma-irradiation but also ROS caused by hydrogen peroxide and menadione than the parent strain BY4741, may suggest that intracellular Mn contributes the response to the damages of ROS in the different trend. *S. cerevisiae* IM3 should be further studies in molecular level as well as DNA sequence or genes expression. Based on these studies, the expecting that the function of Mn(II) ion as an antioxidant will be proved for not only yeast cells but also other organisms.

To sum up, *Saccharomyces cerevisiae* is a unique and promising tool for not only metal bioremediation but also for a study of physiological and detoxification mechanisms. Using *S. cerevisiae* as biomaterial is expected to elucidate the biosorption

and/or bioaccumulation and also ROS scavenging mechanisms. Future studies should be taken into account based on the advantages of yeast processes following the well-documented methodologies.

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LIST OF PUBLICATIONS

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No.	Title of the article	Author(s)	Journal's name, Vol., Pages, and Year	Corresponding chapter
1	Yeast biomass as biomaterial in heavy metal removal: a review	**T.A. Do** M. Furuta	Biocontrol Science (Submitted)	Chapter 1
2	Isolation and characterization of a variant manganese resistant strain of *Saccharomyces cerevisiae*	**T.A. Do** T. Sakai M. Kishida M. Furuta	Biocontrol Science (Submitted)	Chapter 2
3	The role of intracellular manganese in yeast against ROS caused by gamma-irradiation	**T.A. Do** T. Sakai M. Kishida T. Tsuchido M. Furuta	Biocontrol Science (Submitted)	Chapter 3

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