A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Comparative Evaluation of Two Superoxide Dismutases from Redlip Mullet, *Liza haematocheila*: Revealing Their Structural Properties, Immune Response and Antioxidant Defense Mechanism Against Oxidative Stress

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Comparative evaluation of two superoxide dismutases from redlip mullet, *Liza haematocheila*: revealing their structural properties, immune response and antioxidant defense mechanism against oxidative stress

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Summary

In the enzymatic antioxidant system, superoxide dismutases (SODs) are the most important metalloenzymes; they are involved in dismutation of toxic superoxide anions (O_2^-) into hydrogen peroxide (H_2O_2) and oxygen (O_2). Superoxide dismutases (SODs) can be classified into four groups based on the metal residue that binds to the active site: manganese superoxide dismutase (MnSOD), copper/zinc superoxide dismutase (Cu/ZnSOD), iron superoxide dismutase (FeSOD) and nickel superoxide dismutase (NiSOD). Both CuZnSOD and MnSOD are the main SODs which presented in the cytoplasm and mitochondria of cells in the animals, respectively, that help to protect the cells from the adverse effects of excess ROS.

In this study, *Insilico* analysis including multiple sequence alignments, 3D structures, homology analysis and evolutionary relationship were assessed to identify the structural features of mullet CuZnSOD (MuCuZnSOD) and MnSOD (MuMnSOD) using different bioinformatics tools. Transcriptional tissue expression was performed to evaluate the MuCuZnSOD and MuMnSOD expression in 12 different healthy tissues, whereas temporal mRNA expression analysis was conducted using different pathogenic stimulants such as lipopolysaccharide, *Lactococcus garvieae*, and polyinosinic-polycytidylic acid (poly I:C) with blood and liver. Functional characteristics of two SODs were investigated by conventional xanthine oxidase assay and bacterial assay. Also, the peroxidation function of MuCuZnSOD was demonstrated by the cell viability (MTT) assay in the presence of HCO_3^- ions.

The coding sequence of MuCuZnSOD was contained 465 bp and MuMnSOD was possessed 684 bp. The predicted molecular weight of the encoded protein of the MuCuZnSOD was 15.86 kDa, and its isoelectric point was 5.68 while, the MuMnSOD molecular weight was 25.39 kDa, and its isoelectric point was 8.37. Both MuCuZnSOD

and MuMnSOD were detected as intracellular proteins due to the absence of signal peptides in their amino acid sequences. Results of multiple sequence alignments revealed that the MuCuZnSOD contained CuZnSOD domain, Copper/Zinc superoxide dismutase signature 1 (SOD_CU_ZN_1) and Copper/Zinc superoxide dismutase signature 2 (SOD_CU_ZN_2), and MuMnSOD contained iron/manganese superoxide dismutase C-terminal domain (SOD_Fe_C domain), iron/manganese superoxide dismutase N-terminal domain (SOD_Fe_N domain) and the manganese and iron superoxide dismutase signature (Mn/Fe SOD) were highly conserved among their analyzed orthologs. The pairwise alignment results showed that the highest identity was provided by the *Oplegnathus fasciatus* CuZnSOD (94.2 % identity) whereas, MuCuZnSOD gene evolutionary related to the *Oplegnathus fasciatus* according to the phylogenetic tree. Moreover, pairwise alignment revealed that the protein sequence matched to *Larimichthys crocea* with a sequence identity of 95.2%. Phylogenetic tree analysis showed that the MuMnSOD was included in the category of teleosts. The 3D structures of the MuCuZnSOD homo-dimer and MuMnSOD homo-tetramer were identified by the template of the crystal structure of human superoxide dismutase I with 68.83 % identity and human mitochondrial manganese superoxide dismutase with 84.34 % identity, respectively.

Quantitative real-time PCR showed that the highest *MuCuZnSOD* and *MuMnSOD* mRNA expressions were in blood cells. Blood is the main oxygen transporter in the whole body, and it may be the main tissue which subjected to oxidative stress conditions due to direct exposure to the different ROS. The highest expression of *MuCuZnSOD* can be observed at 48 h post injection of poly I:C in the liver and 24 h post-injection in blood. After *L. garvieae* injection, the *MuCuZnSOD* expression was highly up-regulated at 24 h in both liver and blood tissues. The Highest

MuCuZnSOD expression with LPS was observed at 72 h post injection in liver tissue and 6 h in blood. Furthermore, *MuMnSOD* was highly expressed at 24 h and 48 h post-injection of *L. garvieae* in blood and liver whereas, the highest *MuMnSOD* mRNA expression level was observed 24 h and 6 h post-injection with LPS in the liver and blood, respectively. However, the modulation patterns of both *SOD*s against pathogenic stimulants in the blood and liver were different. Therefore, it could be suggested that mRNA expression of *MuMnSOD* might be tissue-specific in the presence of different immune stimulants.

The optimum temperature for XOD activity of both recombinant SODs was found to be 25 °C whereas, optimum pH levels for the activity of MuCuZnSOD and MuMnSOD were pH 9 and pH 7, respectively. Relative XOD activity was significantly increased with the dose of rMuMnSOD, revealing its dose dependency. The activity of rMuCuZnSOD and rMuMnSOD was highly inhibited by potassium cyanide (KCN) and N-N'-diethyl-dithiocarbamate (DDC). Also, the MTT assay revealed that rMuCuZnSOD protein has affected to enhance the cell viability by the peroxidation activity in the presence of HCO_3^- ions. Moreover, results obtained from the antibacterial assay explained that both recombinant proteins reduce the growth of gram-positive and negative bacteria. Results of the present study suggest that MuCuZnSOD and MuMnSOD act as antioxidant enzymes and participate in the immune response in red lip mullet.

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1 Introduction

1.1 Red lip mullet, *Liza haematocheila* as a cultured fish

The redlip mullet is a euryhaline fish species, which belongs to the family Mugilidae, inhabiting a wide range of salinities [1,2]. It is widely distributed in the coast of Korea, Japan, China, and Taiwan. *Liza haematocheila* is an amphidromous species that freely migrates between freshwater and marine water, although it lives most of its life span in the brackish water [3,4]. Evaluating the migration of amphidromous species would provide fundamental knowledge for studies related to fish migration and effective fishery management [5].

The redlip mullet is one of the most valuable fish species contributing to the coastal fishery sector in the Republic of Korea [6]. However, the mullet yield harvested in the wild has declined in recent years due to overhunting and destruction of their habitats [7]. Therefore, it has been focused on wide culturing of mullets as an aqua crop to meet the demand for consumption and contributed for 8% of the total yield of cultured fish in the Republic of Korea in 2012. Redlip mullet is mainly cultured in the west coast of South Korea [8].

Long-term culturing of redlip mullet under an intensive culturing system with poor management have caused widespread of pathogenic diseases and high mortality. Previous research findings reported that *Myxobolus* sp. and *Amyloodinium* sp. causedfor infectious disease conditions in mullet cultivation in South Korea [9,10]. The outbreak of green liver syndrome which developed by *Lactococcus garvieae* was responsible for mass mortality and serious economic loss in cultured red lip mullets, that has been investigated in the past few years [11]. Moreover, harsh environmental

conditions and the presence of environmental pollutants, including hazardous toxin substances and heavy metals may increase oxidative stress in the fish, which leads to invading of pathogenic microorganisms [12,13].

1.2 Reactive oxygen species, oxidative stress and antioxidants

Oxygen is an essential component which is responsible for the cellular respiration of aerobic organisms. The oxygen present in the atmosphere interacts with different challenges; the most important one is the generation of oxygen-derived free radicals or reactive oxygen species (ROS). Free radicals can be defined as any chemical species that contain one or more unpaired electrons [14]. Unpaired electrons generated during the aerobic metabolism of cells are freely accepted by oxygen to form ROS, including superoxide radicles ($O2\cdot$), hydroxyl radicals ($OH\cdot$) and singlet oxygen [15]. The activity of ROS in the animal body mainly depends on the presence of the conditions in internal environment of the body such as ROS concentration, existence, including location, type, proximity and time [16]. The previous study suggested that cell signalling pathways were mediated by ROS and their reaction products as intermediates of cell signalling, thus involved in molecular responses followed by cell growth, development, differentiation, and apoptosis [17].

Oxidative stress is the destruction of balance between the generation of ROS (free radicals) and antioxidant defence mechanism, which may cause for serious tissue damage [18]. ROS is one of the most important factors to initiate reactions related to oxidative stress. Any oxidative substance contains an ability to induce oxidative stress under harsh environmental conditions. Therefore, excessive production of ROS interferes the redox balance and creates an oxidative stress condition that is responsible

for promoting the inflammatory reactions and serious cellular injuries, including peroxidation of proteins and lipids, DNA and RNA damage [19–21].

The activity of ROS in the body has a relationship with its existing environment [22]. Different types of apoptosis is physiologically induced by ROS. Therefore, the formation of ROS is very important for embryo development under normal condition. Moreover, the growth factors essential for the live cells are stimulated by ROS and eliminates inactive proteins via oxidation process, playing a specific role in regulating the general physiological state in different organelles [23]. In the cell, mitochondria is the main organelle which is highly responsible for the ROS generation by uncoupling of electron transport chains and electron leaks [24,25]. Furthermore, other cellular components also synthesis ROS including plasma membrane surface, cytosolic enzymes and endoplasmic reticulum binding enzymes; stimulated multi-enzyme system contained cytochrome oxidase, xanthine oxidoreductase and inflammation associated synthase. Inflammation, hyperoxia and antioxidant defence damage are lead to the oxidative stress of body tissue [16,26].

ROS are highly reactive oxidants that directly harm to the cell structures, and protein, lipids and nucleic acids are directly damaged by ROS as well as ultimately, it leads to cell death through different pathways [27,28]. Lipid peroxidation stimulates sphingomyelinase and releases large amounts of ceramide to induce cell death [29,30]. Protein oxidation and nitrosylation processes, including nitration, nitrotyrosine formation and carbonylation can be initiated by excess ROS amount. Therefore, the biological activities and synthesis of different enzymes cause to significant cellular malfunction. Furthermore, accumulation of high level of ROS adversely effects on oxidized nucleic acids that associate with a breakdown of DNA strands, thus including

necrosis or apoptosis of cells [31,32]. However, aerobic organisms contain a different type of antioxidant that regulate the redox balance and combat against ROS.

The antioxidant defence system is divided into enzymatic antioxidants and non-enzymatic antioxidants under the evolutionary process. In the enzymatic antioxidant defence system, superoxide dismutases (SODs) are the key enzymes; they are associated in dismutation of superoxide anions (O_2^-) into hydrogen peroxide (H_2O_2) and oxygen (O_2) [33,34]. Further breakdown of H_2O_2 into a non-toxic component, water, is catalyzed by catalase (CAT), peroxiredoxin (PRX), glutathione reductase (GSR), glutathione S-transferase (GST), and glutathione peroxidase (GSH-PX) [35]. Thus, these antioxidants are essential for the maintenance of ROS by converting them into non-toxic compounds [36].

1.3 Superoxide dismutase

The key defence mechanism against ROS was discovered by McCord and Fridovich in 1969. It has been reported as dismutation process of superoxide radicals by erythrocuprein protein and suggested the name superoxide dismutase (SOD). Superoxide dismutases (SODs) are metalloenzymes that are responsible for the dismutation of harmful superoxide anions (O_2^-) into hydrogen peroxide (H_2O_2) and oxygen (O_2) [34,37]:

$$2O_2^- + 2H \rightarrow H_2O_2 + O_2$$

H_2O_2 is converted by other antioxidants into harmless product water (H_2O). The combined activity of SODs and other antioxidants like CAT, PRX and GSR have maintained the concentration of O_2^- anions and H_2O_2 [38]. SODs are ubiquitous proteins to all types of living organisms. SODs are categorized into four separate groups based on the metal bound at the active site: CuZnSOD, MnSOD, FeSOD, and NiSOD.

In general, CuZnSODs exist as a homodimeric structure, and molecular mass of each monomer is 14-33 kDa. The catalytic activity of CuZnSOD is resistant to chemicals (4% SDS and 8 M urea) and physical treatments (heating, freezing, freeze-thaw cycles) as well as they are brokendown by proteinase K enzyme [39]. The antioxidant function of CuZnSOD is usually inhibited by inhibitory factors such as H_2O_2, cyanide, azide and diethyldithiocarbamate [40]. The CuZnSODs are normally found in diverse locations in various animal cells such as cytoplasm, mitochondrial inter-membrane space, nucleus, lysosome, peroxisome and extracellular matrix [34,41]. The CuZnSODs are divided into two distinct types; extracellular CuZnSOD and intracellular CuZnSOD encoded by two genes are identified in eukaryotic organisms [42]. The homotetrameric extracellular CuZnSODs of mammals are different from others. They also indicate some structural and functional differences from their cytoplasmic forms, including differences in domain architecture contained in the amino acid sequence, reactions with antibodies and extracellular localization in the presence of heparin on the cell surface [40].

The CuZnSOD enzyme contains Cu^{2+} ion, which is important for oxidized and reduced alternation and Zn^{2+} ion which is essential to maintain the stability of the enzyme. CuZnSOD has been shown to play an important role in defending the cell against superoxide toxicity and acting as a main respiratory for Cu^{2+} ions virtually in all eukaryotic organisms [43]. Deficiency of Cu^{2+} ion causes to suppress the activation and induction of many disease conditions in animals [44,45]. A previous study suggested that CuZnSOD overexpression enhance the lifespan in Drosophila [46]. Other than the conventional activity of CuZnSOD, catalysing of protein nitration is another function [47]. Nitric oxide (NO) mediated vasorelaxation is protected by CuZnSOD present in the blood vessel, hence the vascular tissue activity was maintained

[48]. CuZnSOD is responsible for NO-dependent microbicidal function and the cytokine activation by macrophages in the inflammatory sites [49]. CuZnSOD expression in neuron cells avoids muscle mass loss and function, which occurs in homozygous CuZnSOD knockout mice [50]. The expression level of CuZnSOD differentiates in fish species during heavy metal contamination in the aquatic environment, which creates the fish CuZnSOD a proper biomarker in bioaccumulation studies and eco-toxicological studies [51].

MnSODs are normally presented as two types; mitochondrial and cytosolic forms that contain one metal atom per subunit of 14–30 kDa. The mitochondrial form is homotetrameric and is predominantly found in eukaryotic cells, while the cytosolic form is homodimeric and is found in prokaryotic cells [52]. MnSOD and FeSOD are closely related to the evolutionary process and are similar in structure [53]. However, analysis of MnSOD and FeSOD sequences and crystal structures showed differences between the two types [54]. It helped to predict the metal cofactor, stability of native, kinetic properties and mutant enzymes. Further difference between the two enzymes is the sensitivity to various inhibitory factors. FeSOD activity is inhibited by H_2O_2 and is resistant to KCN, while MnSOD is resistant to both. FeSODs are predominant in prokaryotes, algae, and plant chloroplasts [33,54–56]. NiSODs have been recognized recently in very few organisms which presented as homohexamers composed of ~13 kDa subunits [56]. Only less number of research studies have been conducted on NiSOD andmost of them were based on aerobic soil bacteria; Streptomyces [57].

MnSODs are essential antioxidant enzymes that protect the organism from adverse effects of excess ROS and a wide range of stress-induced disease conditions. Moreover, MnSOD is the only antioxidant, which is important for the survival of life under the different physiological states in an aerobic environment [58,59]. This critical

activity might be occurred due to the location of MnSOD in the mitochondria. MnSODs are nuclear-encoded antioxidants that are highly regulated. MnSOD expression could be regulated at multiple levels, from transcription and translation to post-translational modifications. MnSOD expression and activity are modulated by different extracellular and intracellular factors [60]. Many researchers found that the expression of mitochondrial MnSOD has a relationship with developing cancer and other diseases [61]. Furthermore, it has been indicated that expression of MnSOD could be upregulated by lipopolysaccharide (LPS) [62], ROS, vascular endothelial growth factor (VEGF) [63], and cytokines [64]. After injection of *V. harveyi* into the *Penaeus monodon*, clear changes in MnSOD expression were observed at different time points [65]. Therefore, MnSOD acts as an immune responsive anti-oxidative protein in the host defence system.

1.4 Objectives of the study

In this study, CuZnSOD and MnSOD were identified from the constructed redlip mullet, *Liza haematocheilia* cDNA database and characterized their structural and functional properties. The main objectives of this study were to evaluate the potent antioxidant function and antibacterial activity of the mullet CuZnSOD and MnSOD through functional assays and to compare the gene expression of healthy tissues and those stimulated with different types of pathogenic stimulants.

2 Materials and Methods

2.1 Experimental fish rearing and tissue collection

Healthy mullet fish were purchased from Sang deok fishery center in Hadong, Republic of Korea. The fish were acclimatized using laboratory aquarium tanks at 20 °C for seven days before the experiment. The tissue expression analysis was performed using five fish (average body weight and average length were 100 g and 24cm, respectively). The guidelines for the health and welfare monitoring of the fish were followed throughout the experiment [66]. Tricaine mesylate-MS222 (40 mg/L) was used as a fish anesthetizer. The fish blood was collected using heparin sodium salt (USB, USA) coated sterile syringes. Then immediately, blood was centrifuged and the peripheral blood cells were separated at 3,000 × *g* for 10 min at 4 °C. Other than blood, tissues from head, kidney, liver, stomach, spleen, muscle, gill, skin, intestine, brain, and heart were collected and snap-frozen in liquid nitrogen and stored at −80 °C.

2.2 Construction of cDNA database of redlip mullet

Redlip mullet cDNA database was constructed by the *de novo* assembly. Fish were purchased from the place mentioned above and, average weight and length were also the same as the previous one. The fish were also acclimatized according to the previously described procedure. In brief, blood, head kidney, liver, skin, stomach, heart, spleen, gill, kidney, brain, eye and intestine of 5 fish were used for RNA extraction. Then, the extracted RNA content was sent, and Pacbio platform was used for sequencing at Insilicogen, Republic of Korea.

2.3 Immune challenge experiment for redlip mullet

Immune stimulation experiment was carried out using four groups of fish and each group was consisted with five fish, which has average body weight of 100 g. Group of mullets were injected intraperitoneally using three immune stimulants such as lipopolysaccharide; LPS (1.25 μg/g), polyinosinic:polycytidylic acid; poly I:C (1.5 μg/g) and *Lactococcus garvieae* (1×10^3 CFU/μL) which were suspended in phosphate-buffered saline (PBS). Control fish group was injected by 100 μL of PBS. The peripheral blood and liver tissues were taken from fish mullets at 0, 6, 24, 48, 72 h post-injection, as mentioned in section 2.1.

2.4 Isolation of RNA and cDNA synthesis

Total RNA amount present in the pooled tissues from five mullets was isolated using RNAiso plus (TaKaRa, Japan) and RNeasy spin column (Qiagen, Germany) was used for the clean up (n=5 for tissue distribution and immune challenge). In addition, the purity of RNA was evaluated by 1.5% agarose gel electrophoresis. Then, the RNA concentration of each tissue was measured using μDrop Plate (Thermo Scientific) at 260 nm. Synthesis of first strand cDNA was carried out by PrimeScript™ II 1st strand cDNA Synthesis Kit (TaKaRa, Japan). cDNA synthesis reaction mixture (20 μL) contained 2.5 μg of RNA. Finally, nuclease-free water was used to dilute (40-fold) synthesized cDNA and stored at −80 °C.

2.5 Relative mRNA expression

The relative mRNA expression levels of *MuCuZnSOD* and *MuMnSOD* transcripts were investigated in healthy mullet tissues and immune challenged tissues through Quantitative real-time PCR (qPCR) in a Thermal Cycler Dice™ TP950 (TaKaRa, Japan). The qPCR analysis was carried out using specifically designed

primers (Table 1) and 10 μL of reaction mixture by following the condition described in our previous report [67]. The Livak method was used for the calculation of relative transcript levels of samples [68], and the reference gene was Mullet *Elongation Factor 1 alpha* (*EF1α*) (GenBank ID: MH017208). The expression levels of post-challenged tissues were compared with the PBS-injected control group at each corresponding time point.

2.6 *In silico* analysis

Domain structure and signatures of both SODs were identified by the ExPASy PROSITE web tool (http://prosite.expasy.org/), SMART tool (http://smart.embl-heidelberg.de/) and Motif Scan software (http://myhits.isb-sib.ch/cgi-bin/motif_scan). The cellular localization and N-linked glycosylation sites of MuCuZnSOD and MuMnSOD were predicted using PSORT II server (https://psort.hgc.jp/form2.html) and NetNGlyc software (http://www.cbs.dtu.dk/services/NetNGlyc/) respectively. The ProtParam web tool (http://web.expasy.org/protparam/) was used to calculate the molecular weight, isoelectric point and instability index of both SODs. Cysteine sites of MuCuZnSOD were identified using the DiANNA 1.1 tool (http://clavius.bc.edu/~clotelab/DiANNA/). Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) was used to design the multiple sequence alignments. Sequence identity and similarities were observed using EMBOSS Needle web tool (https://www.ebi.ac.uk/Tools/psa/emboss_needle/). Neighbour-joining trees were constructed by the Neighbor-Joining method using MEGA software (version 5). The structures of MuCuZnSOD and MuMnSOD were recognized by SWISS-MODEL online tool (https://swissmodel.expasy.org/interactive) and visualized by PyMOL software.

2.7 Preparation of the expression vector *pMAL*-c5X

The coding sequences (CDS) of the cDNA fragments were amplified using gene-specific cloning primers (Table 1). The size of the MuCuZnSOD and MuMnSOD amplicons were 465 bp and 684 bp respectively, and they were isolated from a 1% agarose gel using a gel purification kit (BIONEER, Korea). EcoRI and HindIII were used as restriction enzymes to digest the amplicons and *pMAL*-c5X plasmids containing the ampicillin marker. Digested products were ligated using Mighty Mix DNA Ligation Kit (TaKaRa, Japan). Then, the recombinant plasmids were transformed into *Escherichia coli* competent cells (DH5α). Transformed DH5α cells were grown on an LB-ampicillin plate at 37 ℃ overnight. Successive clones were recognized, and the sequence was verified by sequencing (Macrogen, Korea). The sequence confirmed plasmid was transformed into *E. coli* ER2523 competent cells for expression of the relevant proteins.

Table 1. **Description of primers**

Primer Name	Primer sequence (5'-3')
MuCuZnSOD forward (*EcoR I*)	gagagagaattcATGGTGCTGAAAGCTGTTTGTGTG
MuCuZnSOD reverse (*Hind III*)	gagagaaagcttTTACTGGGTGATGCCAATGACTCC
MuMnSOD forward (*EcoR I*)	gagagagaattcATGAACATGCTGTGCAGAGTTGGTC
MuMnSOD reverse (*Hind III*)	gagagaaagcttCTACTTTTTGGCAATCTGAAGACGGTCG
MuCuZnSOD forward (qPCR)	CATGGCTTCCATGTCCATGCCTTT
MuCuZnSOD reverse (qPCR)	GGCAACATTATCGGATCCTGCAGTC
MuMnSOD forward (qPCR)	TGTCTGCTGCTACCGTTGCA
MuMnSOD reverse (qPCR)	GGGCCGCACATTCTTGTACTGAA
MuEF1α forward (qPCR)	CCCTGGTCAGATCAGTGCTGGTTAT
MuEF1α reverse (qPCR)	AGCGTCGCCAGACTTTAGGGATTT

2.8 Overexpression and purification of recombinant proteins

Recombinant MuCuZnSOD (rMuCuZnSOD) and MuMnSOD (rMuMnSOD) proteins were overexpressed as a fusion protein with a maltose binding protein tag in *E. coli* ER2523 cells. Transformed *E. coli* ER2523 cells were grown in LB rich ampicillin medium (500 mL) which contained 100 mg mL^{-1} ampicillin and 0.2% glucose. The culture was incubated at 37 °C and 200 rpm in a shaking incubator. On the following day, the seed culture was inoculated into LB rich ampicillin medium and incubated at 37 °C and 200 rpm until the OD_{600} reached 0.5. Isopropyl-b-thiogalactopyranoside (IPTG; 0.25 mM) was added into the culture medium to induce the expression of the protein at 20 °C and 200 rpm for 10 h. Next, the induced cell culture was pre-chilled for 30 min on ice and harvested at 3500 × *g* for 30 min at 4 °C. Harvested cells were re-suspended in the column buffer, which contained 20 mM Tris-HCl, 200 mM NaCl, pH 7.4, and stored at -20 °C overnight.

On the following day, the suspension of the cells was thawed, and 1 mg mL^{-1} of lysozyme was added. Afterwards, the lysed cell sample was sonicated, and the cell lysate was centrifuged at 20,000 × *g* at 4 °C for 30 min. The supernatant was separated and purified on a column with amylose resin (New England Biolabs, USA). The purified rMuCuZnSOD and rMuMnSOD were eluted using elution buffer composed of column buffer and 10 mM maltose. Simultaneously, overexpression and purification of the recombinant MBP (rMBP) were completed. Concentrations of purified rMuCuZnSOD, rMuMnSOD and rMBP were measured using the Bradford technique [69]. Efficacy of protein purification was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% SDS-PAGE) under reducing conditions.

2.9 Functional assays for rMuCuZnSOD and rMuMnSOD

2.9.1 Xanthine/xanthine oxidase (XOD) assay

Superoxide scavenging activity of rMuCuZnSOD and rMuMnSOD were measured by the XOD assay following the previously described procedure [67,70]. The antioxidant function was quantified by the conventional spectrophotometry. A total volume of 160 μL reaction mixture was prepared using 0.1 M of glycine-NaOH buffer (pH 9), 3mM ethylenediaminetetraacetic acid (EDTA), 0.75 mM nitroblue tetrazolium (NBT), 0.15% bovine serum albumin (BSA), 3 mM xanthine, rMuCuZnSOD and rMuMnSOD, and incubated at 25 °C for 10 min. Then, 6 mU xanthine oxidase was introduced to the reaction mixture and incubated at 25 °C for 20 min. The microplate reader (Multiskan GO, Thermo Scientific, Finland) was used to quantify the absorbance at OD_{560} in each sample. Also, recombinant MBP (rMBP) sample and blank sample were used as positive control and negative control, respectively.

2.9.2 Biochemical properties of rMuCuZnSOD and rMuMnSOD

Biochemical properties of rMuCuZnSOD and rMuMnSOD were determined using XOD assay mentioned in section 2.9.1. The XOD assay was carried out using three buffers in nine different pH values including citrate (pH 3, 4, 5), phosphate (pH 5, 7, 8) and glycine-NaOH (pH 9, 10, 11). The activity of rMuCuZnSOD and rMuMnSOD were assessed at different temperatures from 5 to 85 °C at different intervals to identify the optimum temperature. Then, XOD assay was again performed to assess the effect of recombinant protein concentration on the SOD activity and different rMuCuZnSOD, rMuMnSOD and rMBP amounts were used such as 10, 20, 30, 40, 50, 60, 70 and 80 μg. Finally, the effect of inhibitory factors (6.25 mM of potassium cyanide (KCN), sodium azide (NaN_3), N-N'-diethyl-dithiocarbamate (DDC)

and ethylenediamine tetraacetic acid (EDTA) on the superoxide scavenging activity of rMuCuZnSOD and rMuMnSOD were evaluated according to our previous study [67].

2.9.3 Peroxidation activity of rMuCuZnSOD by cell viability assay

A Cell viability assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay; MTT) was conducted to evaluate the cell survival rate during oxidative stress condition in the presence of rMuCuZnSOD. Kidney epithelial (Vero) cells from African green monkey were cultured using RPMI 1640 culture media in a 12-well plate in a 5% CO_2 incubator at 37 °C. The cell growth medium was contained 10% of FBS, 100 U/mL of penicillin and 100 mg/mL of streptomycin. The rMuCuZnSOD and rMBP protein were used to treat the cells in the presence of 20 mM $NaHCO_3$. Then, 400 μmol of H_2O_2 was added to the cells and incubated at a 1×10^5 cells/mL of density for 24 h. Finally, the cell viability was measured using the microplate reader (Multiskan GO, Thermo Scientific, Finland) at an absorbance of 540 nm [71,72].

2.9.4 Antibacterial activity of rMuCuZnSOD and rMuMnSOD

Antibacterial activity of rMuCuZnSOD and rMuMnSOD were assessed using gram-positive bacteria (*Micrococcus luteus* and *L. garvieae*) and gram-negative bacteria (*E. coli*) [73,74]. Different bacterial media were used to culture the bacteria. *M. luteus*, *L. garvieae* and *E. coli* were grown in BHI, BHIS (1.5 % sodium) and LB media to mid-logarithmic phase respectively. The cultured bacteria were then collected at 5000 x *g* of centrifugation for 20 min at 4 °C. Collected bacterial pellets were washed using PBS and re-suspended in fresh media, while concentrations were 2×10^7 cells/mL. A 96-well plate was used to seed the bacterial cells and then cells were separately treated using two concentrations of recombinant proteins (100 μg/mL and 200 μg/mL) at their optimal temperatures for 10 h. Finally, the microplate reader

(Multiskan GO, Thermo Scientific, Finland) was used to measure the absorbance at 600 nm in 1 h interval for 10 h.

2.10 Statistical analysis

All the data in the assays were reported as means ± SD of triplicate measurements. Statistical significance of each assay was identified by unpaired, two-tailed t-test for calculation of P-values using the GraphPad software (GraphPad Software, Inc., USA).

3 Results and Discussion

3.1 Structural features of MuCuZnSOD and MuMnSOD sequences

The complete coding sequences (CDS) of MuCuZnSOD and MuMnSOD were identified from the redlip mullet cDNA transcriptomic database by homology screening. The complete cDNA sequences of MuCuZnSOD and MuMnSOD were deposited in NCBI GenBank under the accession numbers of MK860772 and MH018137 respectively. MuCuZnSOD complete cDNA sequence comprised 1550 bp, including a 130 bp 5' untranslated region (UTR), a 465 bp CDS that encodes a 154 amino acids, and a 955 bp 3'UTR, while The 5' untranslated region (UTR) of the MuMnSOD contained 230 bp; the CDS contained 684 bp which encoded 227 amino acids; and the 3'UTR contained 437 bp (Figure 1).

(A)

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                                                        ATTTCTAATC      -130
TGATTGGTTGTTTCGTAAAACAGGCCCGCCCACTAGCCACAAATATGGTTGGTTGTCACT      -120
CTACTAGCAAGAGAGCACGCCACATTAAGTTGGTCGAGCTGTGAAAGTACTGCTGCAAAG      -60
ATGGTGCTGAAAGCTGTTTGTGTGTTGAAAGGAGCTGGAGAGACCAGCGGGACAGTTTTT       60
 M  V  L  K  A  V  C  V  L  K  G  A  G  E  T  S  G  T  V  F       20
TTTGAGCAGGAGGATGCCTCTTCCCCTGTGAAGTTGACGGGAGAAATCAAAGGTCTTACC      120
 F  E  Q  E  D  A  S  S  P  V  K  L  T  G  E  I  K  G  L  T       40
CCTGGTGAGCATGGCTTCCATGTCCATGCCTTTGGGGATAACACAAACGGATGCATCAGT      180
 P  G  E  H  G  F  H  V  H  A  F  G  D  N  T  N  G  C  I  S       60
GCAGGCCCTCACTTCAATCCCCACAACAAGAATCACGCTGGCCCCAATGATGCAGAAAGA      240
 A  G  P  H  F  N  P  H  N  K  N  H  A  G  P  N  D  A  E  R       80
CACGTTGGAGACCTGGGCAACGTGACTGCAGGATCCGATAATGTTGCCAAGCTAGACATC      300
 H  V  G  D  L  G  N  V  T  A  G  S  D  N  V  A  K  L  D  I      100
ACAGACAAGATAATCACCCTCACGGGCCCCGACTCCATCATTGGCAGAACCATGGTGATC      360
 T  D  K  I  I  T  L  T  G  P  D  S  I  I  G  R  T  M  V  I      120
CACGAGAAGGCAGATGACCTTGGAAAAGGAGGAAACGAGGAGAGCCTGAAGACCGGTAAT      420
 H  E  K  A  D  D  L  G  K  G  G  N  E  E  S  L  K  T  G  N      140
GCAGGTGGACGTCTGGCCTGTGGAGTCATTGGCATCACCCAGTAA                     465
 A  G  G  R  L  A  C  G  V  I  G  I  T  Q  *                      154
AGAAAACGCCAGAGCTCAAAGCACTGAAAACGCTTTTCCCCACAGCACTTAATGAGACCA      525
ACATAGCTACTTGATGTGACAGTTTGCCCTTTTTTCAATTCTCTACGCCGTTTTGTCAGA      585
AGAGTAGATGAGCCACTCTTTGCCCTGTCAGTTCCTAATGACAGTTGTATGTAAGAGTTT      645
ATATGTCTGCAGATTAGTTTTGTTCCCAAAGAATTGGTAACGCACAAGTAATAACCAGAT      705
GTGTAAAAAAGTTACAAGATGAATAAATTGTTTATCTGAATCATGTGAGTAGTATGTCAT      765
TTAAAGTGTGTGTGTGTGACGTTAACGGTACAAACAAACCTGGATAAAGTTCAGCATTTC      825
TTTTCTAGGTTGTGACTAAAGTTGGACGTTACTGCAGTTAAATTGTATTTTAGCAGTGCT      885
GTTTAGGGATGCGTGATAGTATGGCTTACTGACACGGGGTGTACCTTTGTTTAATCTTAC      945
TAATTAACTAGAATATCAGCTTCTTCAATGGTGTTTGCTTCCCCTACTGTGGCAAATATT     1005
ATGAATGTGTTGCACCTGCTATGGTGAAAATATGGCAGGTTTGTGGGATGATGTCAACCA     1065
CTAGTGGTCTTCCTGAGATGATGATCTGTACAGTCCTAGTCCACGTGAAACCTGGTTGAA     1125
GGTAAGGTAAAATAACGTAGAAAGACGCAGTTTAAGGTCTGATGGTACCATGAAGAATAA     1185
CGACATTTTGGTTGTTGATGGATGCACACGGGACAACAAAGACATACATGTGAATTAACC     1245
GTTTTTCACGAGATAAAATATTTGAACAAGTACACATTCAGAAGCAGAGAGTCCTTTGTT     1305
CAAACAATAGCTTTGATCAACTTCACGCTGTATTAACCCCTATGTTCCTCTAGAGGGCGA     1365
TGCTACTCCTAATATTTCAAATTGCAACTGATTCTACTCAGTTGCTTCATCACAG           1420
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(B)

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          CTATACTTTCGCAACACGTCTTTATATATTTTATTTTTTAGTATTTCTGT      -230
GATGAGTATCACTTCCATGAGTGTCCGTTGGGAGTTTATCTATTCAACGCACACTCTCAT      -180
AAACACCCCCCCCGCCGCATGGTAATGGTACGGTCGCCCAGCTCTGTCAACTTGAAATTGC     -120
AGACTTCAAGGGCAGTTACCAGCGTGTCTACTCTGTTCTGTCTACCCATTGAGCATTATC      -60
ATGAACATGCTGTGCAGAGTTGGTCTGATACGCAGGTGTGCGGCCAGCCTGAATGTAACT       60
 M  N  M  L  C  R  V  G  L  I  R  R  C  A  A  S  L  N  V  T       20
ATAAACCAGGTAGCTGCATCAAGGCAGAAGCACACGCTTCCTGACCTGACGTATGACTAT     120
 I  N  Q  V  A  A  S  R  Q  K  H  T  L  P  D  L  T  Y  D  Y       40
GGTGCCCTGGAGCCCCACATCAATGCAGAGATAATGCAGCTGCACCACAGCAAGCATCAT     180
 G  A  L  E  P  H  I  N  A  E  I  M  Q  L  H  H  S  K  H  H       60
GCAACATATGTCAATAACCTCAACGTGACGGAGGAGAAATATCAGGAGGCGCTCGCAAAG     240
 A  T  Y  V  N  N  L  N  V  T  E  E  K  Y  Q  E  A  L  A  K       80
GGAGATGTGTCGACTCAGGTTGCCCTTCAGCCTGCCCTGAAGTTTAACGGAGGCGGCCAC     300
 G  D  V  S  T  Q  V  A  L  Q  P  A  L  K  F  N  G  G  G  H      100
GTTAACCACACTATCTTCTGGACAAACCTCTCTCCAAATGGTGGAGGCGAGCCACAGGGG     360
 V  N  H  T  I  F  W  T  N  L  S  P  N  G  G  G  E  P  Q  G      120
GAACTAATGGAGGCCATCAAGCGGGACTTCGGCTCCTTCCAGAAGATGAAAGAGAGGATG     420
 E  L  M  E  A  I  K  R  D  F  G  S  F  Q  K  M  K  E  R  M      140
TCTGCTGCTACCGTTGCAGTGCAGGGCTCCGGCTGGGGATGGCTGGGTTACGAGAAGGAG     480
 S  A  A  T  V  A  V  Q  G  S  G  W  G  W  L  G  Y  E  K  E      160
AGTGGGAGACTTCGCATTGCCGCTTGTGCTAACCAAGATCCTCTGCAGGGCACGACAGGT     540
 S  G  R  L  R  I  A  A  C  A  N  Q  D  P  L  Q  G  T  T  G      180
CTCATTCCTCTCCTTGGCATTGACGTATGGGAGCATGCCTACTACCTTCAGTACAAGAAT     600
 L  I  P  L  L  G  I  D  V  W  E  H  A  Y  Y  L  Q  Y  K  N      200
GTGCGGCCCGACTATGTTAAGGCTATCTGGAACGTAATCAACTGGGAGAACGTGAGCGAC     660
 V  R  P  D  Y  V  K  A  I  W  N  V  I  N  W  E  N  V  S  D      220
CGTCTTCAGATTGCCAAAAAGTAG                                        684
 R  L  Q  I  A  K  K  *                                         227
ACGCCCAATAATCAAACATACCCCTTCACAAATAACTTTGAGTTTTGCACACACTGAGTA     744
TCTAATTCAGCAGCAGACATGTTCCCTTTCAGATTCAAGACAGTTCAGTGCTACTAAAGT     804
TGATTAAATTGGCTTTAAAACACTCTAAACTGGTGCAGATTATATTGTCTTTATTGTCAT     864
GTATCGCAGGTTGAAACAAGCATAAGTTATTTACTAGTGCTTTTAATCTCTGGCCTGTTC     924
CTAACCACCTGATTATTTTCAAAACGCACAAAAAAATGGTCACCTTGAATGTTAAAGCTG     984
ACTTTTGTTATACACAAGGTAACAGGTGGCATACTAGATAAGCAAGCGTATGGTCTGTGA    1044
GCCGTGTCTTGGTCACAATAAATACAATGTTATTGAATCCCGTGTTTTTGATGTATTATA    1104
ATAAAATAATGAGATTT                                               1164
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Figure 1. Sequence analysis of MuCuZnSOD (A) and MuMnSOD (B).
The deduced amino acid sequences are bolted in purple colour and, starting and stop codons bolted in red colour in both SODs. The CuZnSOD domain is shaded in light grey whereas SOD_CU_ZN_1 signature is showed by the green line and the SOD_CU_ZN_2 signature indicated by yellow line (A). The SOD_Fe_N domain of MuMnSOD sequence is shaded light grey, and the SOD_Fe_C domain is highlighted by a dark grey shading. The HAMP domain and manganese and iron superoxide dismutase signature are underlined by red and green, respectively (B).

The predicted molecular mass of the MuCuZnSOD and MuMnSOD were 15.86 kDa and 25.39 kDa and their theoretical *p*I values were 5.68 and 8.37, respectively. The high *p*I value is related to the Cu^{2+} ion binding activity of histidine in MuCuZnSOD [75]. However, low pH levels (<4) lead to a decrease in the Cu^{2+} ion binding affinity. Because of the high *p*I value of MuCuZnSOD protein, the Cu^{2+} ion binding affinity reduction is not affected by the pH [75,76]. The instability index of 13.61 and 34.99 were identified

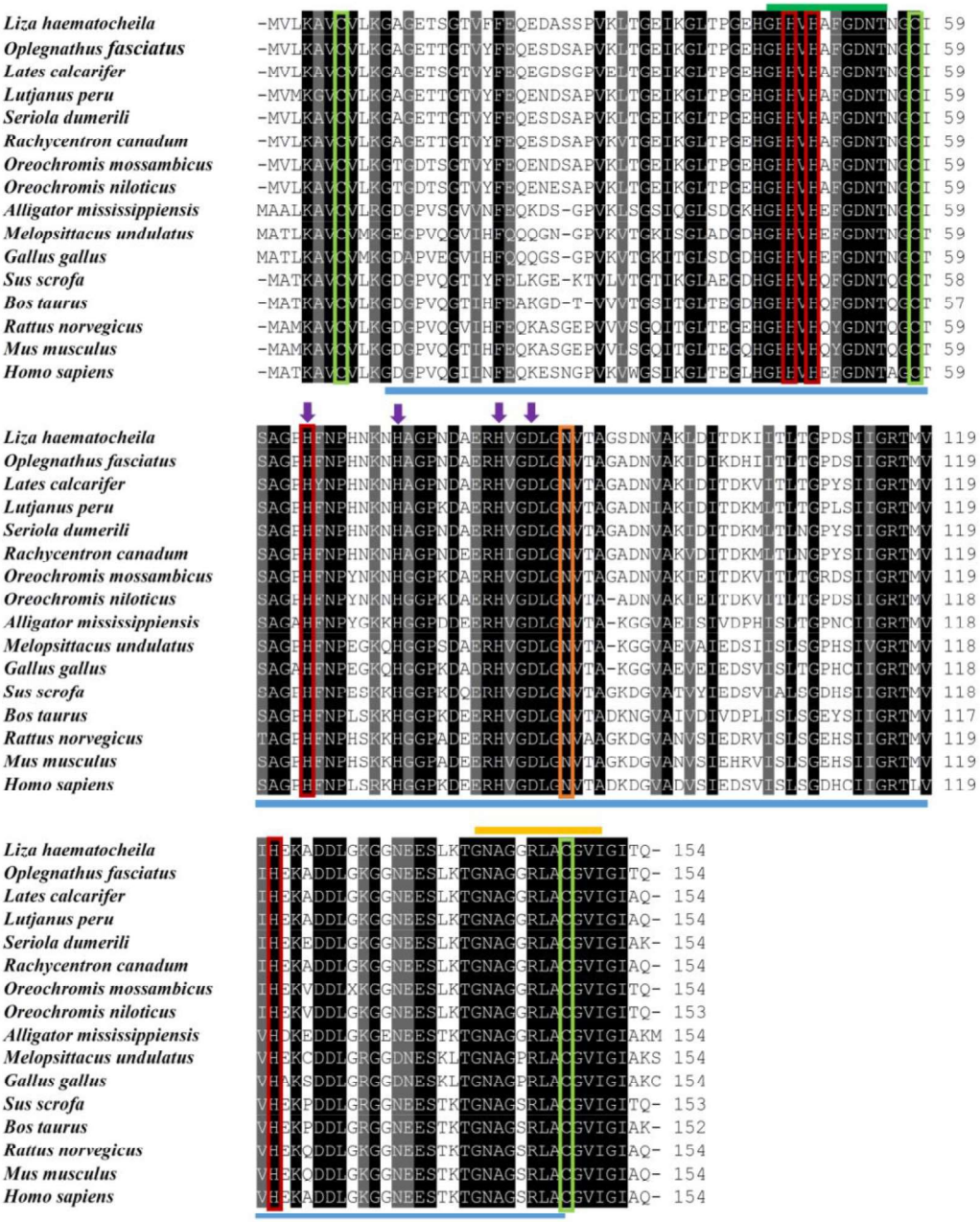
in MuCuZnSOD and MuMnSOD, respectively, suggesting that the encoded proteins of both SODs were stable [77].

The amino acid sequence analysis indicated that both SODs did not contain signal peptides. Therefore, these SODs might be detected as intracellular proteins. The PSORT II server results suggested that MuCuZnSOD was presented in the cytoplasm and MuMnSOD was presented in the mitochondria. Subcellular localization studies have reported that CuZnSODs are identified in the main organelles including the cytoplasm, nucleus and interspace of the mitochondrial membrane, while MnSODs highly exist in the mitochondrial matrix, where the ROS are highly generated [78]. The mitochondrial matrix is important as the site that generates energy for cells. Thus, high amounts of ROS may be released in the electron transport system [79]. MnSOD is the principle antioxidant enzyme present in the mitochondrial matrix of eukaryotic organisms. Cytosolic forms of MnSOD isoforms have been found throughout the cellular compartments in various organisms displaying their antioxidant capacity. However, mitochondrial MnSOD and cytosolic CuZnSOD could play a distinct function in protecting cellular components against ROS in eukaryotic organisms [79].

The MuCuZnSOD protein sequence contained an N-glycosylation site (^{87}NVTA^{90}), which suggested that the protein might be a glycoprotein [70,80]. The results obtained from the PROSITE web server has shown that CuZnSOD domain at 11-147 amino acids, two signatures (^{45}GFHVHAFGDNT^{55} and ^{139}GNAGGRLACGVI^{150}) and three cysteine residues (C^{7}, C^{58}, and C^{147}) were in the MuCuZnSOD sequence. Predicted three cysteine residues contribute to generating an intrachain disulfide bond. Therefore, CuZnSOD proteins can produce a homodimer and the two subunits in the homodimer are stabilized by the disulfide bond [81]. These cysteine residues are also responsible for stabilizing the CuZnSOD protein under high

temperatures [82,83]. Four different binding sites for Cu^{2+} and Zn^{2+} were predicted, which might be important to mediate the redox reaction and regulate the enzyme stability [84]. The active sites present in the protein sequence of MuCuZnSOD can be named as electro-statistical relevant residues and they might be responsible for detecting the substrates of superoxide anions [85] (Figure 2).

(A)



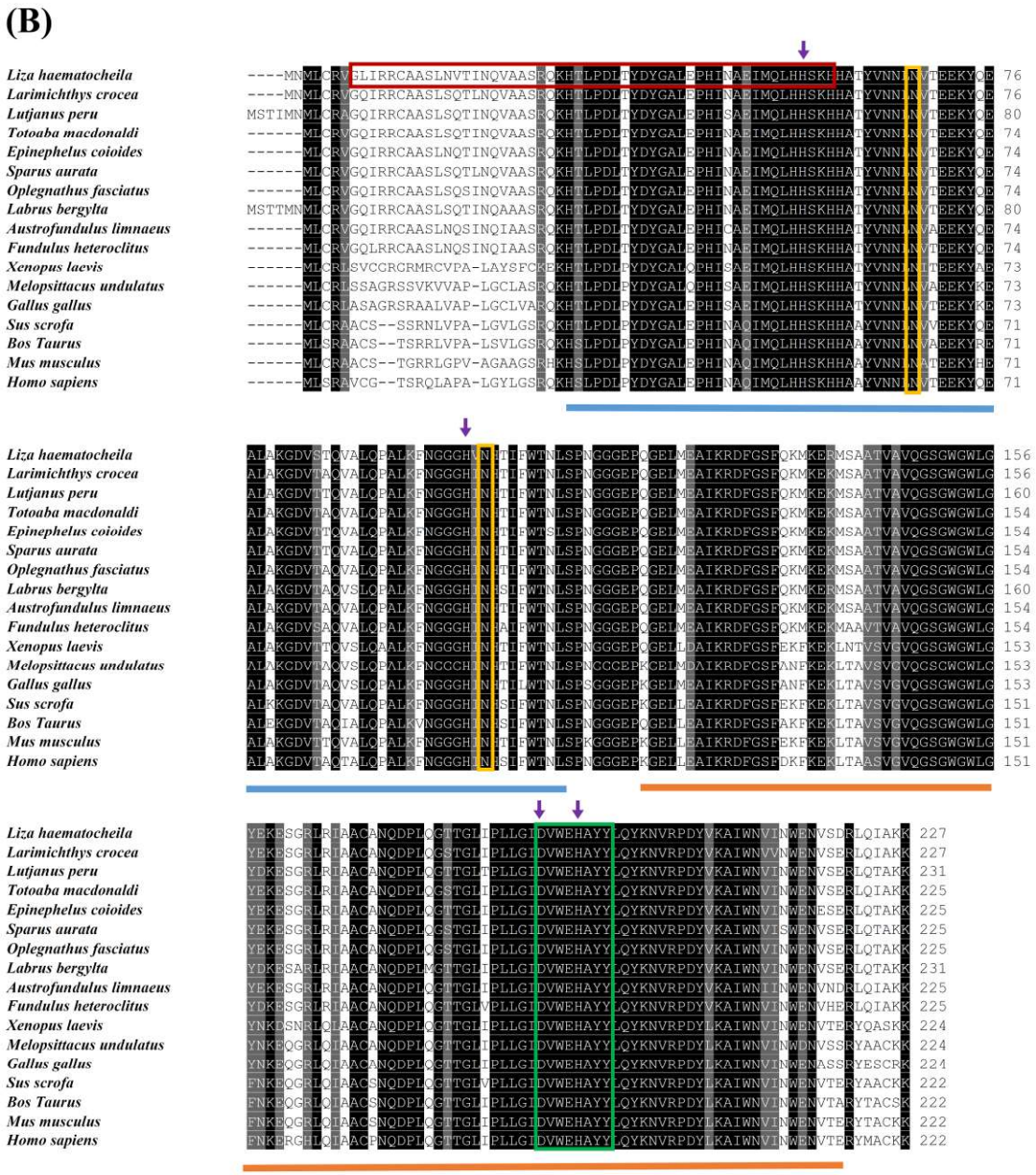


Figure 2. Multiple sequence alignment of MuCuZnSOD (A) and MuMnSOD (B). Identical amino acids are highlighted in black, and partially conserved amino acids are shaded in grey colour in both SODs. (A) CuZnSOD sequence alignment. Red, green and orange color boxes represent the Cu^{2+} binding sites, cysteine residues and *N*-glycosylation site respectively. The Zn^{2+} binding sites also are marked in purple colour arrows. The SOD_CU_ZN_1 signature is showed by the green line and the SOD_CU_ZN_2 signature is indicated by yellow line whereas CuZnSOD domain is marked in the blue line. (B) MnSOD sequence alignment. The SOD_Fe_N domain is marked with a blue line whereas the SOD_Fe_C domain is indicated by an orange line; the HAMP domain is indicated by a red box. Manganese and iron superoxide dismutase signature and N-glycosylation sites are indicated by yellow and green boxes, respectively. Four distinct Mn^{2+} binding sites are shown by four arrows.

MuMnSOD might be a glycoprotein owing to the presence of two *N*-glycosylation sites (^{68}NVTE^{71} and ^{102}NHTI^{105}). Two main domains can be detected in the protein sequence of MuMnSOD, including an iron/manganese superoxide dismutase C-terminal domain (SOD_Fe_C domain) and an iron/manganese superoxide dismutase N-terminal domain (SOD_Fe_N domain). Four distinct binding sites for Mn^{2+} were predicted (H^{55}, H^{99}, $D^{188,}$ and H^{192}), which may be important for its catalytic function [86] (Figure 2). ExPASy PROSITE web tool revealed that the manganese and iron superoxide dismutase signature (Mn/Fe SOD) could be recognized in the protein sequence of the MuMnSOD. SMART web tool identified the HAMP domain, which is mainly found in chemotaxis proteins and histidine kinases as a sensor of bacteria [87] and eukaryotic cells. The HAMP domain may associate with numerous domains including the histidine kinase, the bacterial chemotaxis sensory transducer domain, the protein phosphatase 2C-like domain and the guanylate cyclase domain. The HAMP domain facilitates the transmission of conformational changes from the periplasmic ligand binding domain to the cytoplasmic signal kinase domain and the methyl acceptor domain. Therefore, it regulates the methylation and phosphorylation functions of homodimeric receptors [87–89]. Moreover, conformational changes of the HAMP domain caused a decrease of the kinase enzyme activity due to the binding of MnSOD [90–92]; this adversely affected phosphorylation [93]. Therefore, conformational changes of the HAMP domain presented in the MuMnSOD might be involved in reducing bacterial growth by decreasing the kinase activity of bacteria.

3.2 Analysis of homology and evolutionary relationship

The pairwise alignment results obtained from ClustalW web server highlighted that the highest identity and similarity were provided by the *Oplegnathus fasciatus* CuZnSOD (94.2 % identity and 97.4 % similarity) followed by *Lates calcarifer* (92.9 %

identity and 96.8 % similarity) and *Maylandia zebra* (90.9 % identity and 96.8 % similarity). The *Larimichthys crocea* MnSOD (95.2% identity and 98.8% similarity) followed by *Totoaba macdonaldi* (95.2% identity and 97.8% similarity) and *Epinephelus coioides* (95.2% identity and 97.4% similarity) showed significant high identity and similarity (Table 2).

Table 2. Homology analysis of MuCuZnSOD and MuMnSOD

Organisms (CuZnSOD)	Taxonomy	Identity %	Similarity %	Gaps %	AA
Oplegnathus fasciatus	Teleostei	94.2	97.4	0.0	154
Lates calcarifer	Teleostei	92.9	96.8	0.0	154
Maylandia zebra	Teleostei	90.9	96.8	0.0	154
Seriola dumerili	Teleostei	90.9	96.1	0.0	154
Lutjanus peru	Teleostei	90.3	96.8	0.0	154
Rachycentron canadum	Teleostei	90.3	96.1	0.0	154
Oreochromis niloticus	Teleostei	90.3	96.1	0.6	153
Oreochromis mossambicus	Teleostei	89.6	95.5	0.0	154
Mus musculus	Mammalia	70.1	79.9	0.0	154
Alligator mississippiensis	Reptile	69.9	79.5	2.6	154
Sus scrofa	Mammalia	69.5	76.6	0.6	153
Rattus norvegicus	Mammalia	68.8	79.2	0.0	154
Homo sapiens	Mammalia	68.8	77.9	0.0	154
Bos taurus	Mammalia	67.5	76.0	1.3	152
Melopsittacus undulatus	Aves	65.4	76.9	2.6	154
Gallus gallus	Aves	63.5	75.6	2.6	154
Organisms (MnSOD)	Taxonomy	Identity %	Similarity %	Gaps %	AA
Larimichthys crocea	Teleostei	95.2	98.8	0.0	227
Totoaba macdonaldi	Teleostei	95.2	97.8	0.9	225
Epinephelus coioides	Teleostei	95.2	97.4	0.9	225
Sparus aurata	Teleostei	94.3	97.8	0.9	225
Oplegnathus fasciatus	Teleostei	94.3	97.4	0.9	225
Austrofundulus limnaeus	Teleostei	93.8	96.9	0.9	225
Lutjanus peru	Teleostei	93.1	96.1	1.7	231
Fundulus heteroclitus	Teleostei	92.5	96.5	0.9	225
Labrus bergylta	Teleostei	92.2	95.2	1.7	231
Gallus gallus	Aves	78.4	86.8	1.3	224
Sus scrofa	Mammalia	78.1	86.8	3.1	222
Melopsittacus undulatus	Aves	78.0	86.3	1.3	224
Homo sapiens	Mammalia	77.3	85.2	3.9	222
Mus musculus	Mammalia	76.8	85.5	3.1	222
Xenopus laevis	Amphibia	75.3	86.6	4.8	224
Bos taurus	Mammalia	74.3	83.0	4.8	222

Moreover, MuCuZnSOD and MuMnSOD sequences shared considerable sequence identity and similarity with both SODs of mammals (>65 % identity and >75 % similarity) and birds (>60 % identity and >75 % similarity).

The multiple sequence alignment results showed that the CuZnSOD domain, Copper/Zinc superoxide dismutase signature 1 (SOD_CU_ZN_1), Copper/Zinc superoxide dismutase signature 2 (SOD_CU_ZN_2), *N*-glycosylation site, cysteine residues, Cu^{2+} binding sites and Zn^{2+} binding sites were highly conserved with other CuZnSOD orthologs examined in the current study. However, the sequence alignment of MuMnSOD also revealed that the SOD_Fe_N domain, the SOD_Fe_C domain, *N*-glycosylation sites and the Mn/Fe SOD signature were highly conserved among the other MnSOD orthologs inspected in the present study (Figure 2). The previous literature affirmed that the protein sequences of CuZnSOD and MnSOD are conserved through the evolutionary process, especially the structural domains and the specific active sites [70,94]. However, structural characters evaluated through the analysis of homology suggest that the antioxidant activity and other functions of both SODs might be conserved with its counterparts.

The phylogenetic trees were developed to examine the evolutionary relationship of two SODs by the Neighbor-Joining technique using the protein sequences of known orthologs. Results obtained from the analysis of phylogenetic tree highlighted that the MuCuZnSOD and MuMnSOD were clustered into the teleosts category that provided a basis of origin from the common ancestor [70] and they were divided from other groups. Moreover, MuCuZnSOD gene was closely related to the CuZnSOD of *O. fasciatus* in the evolutionary process (Figure 3).

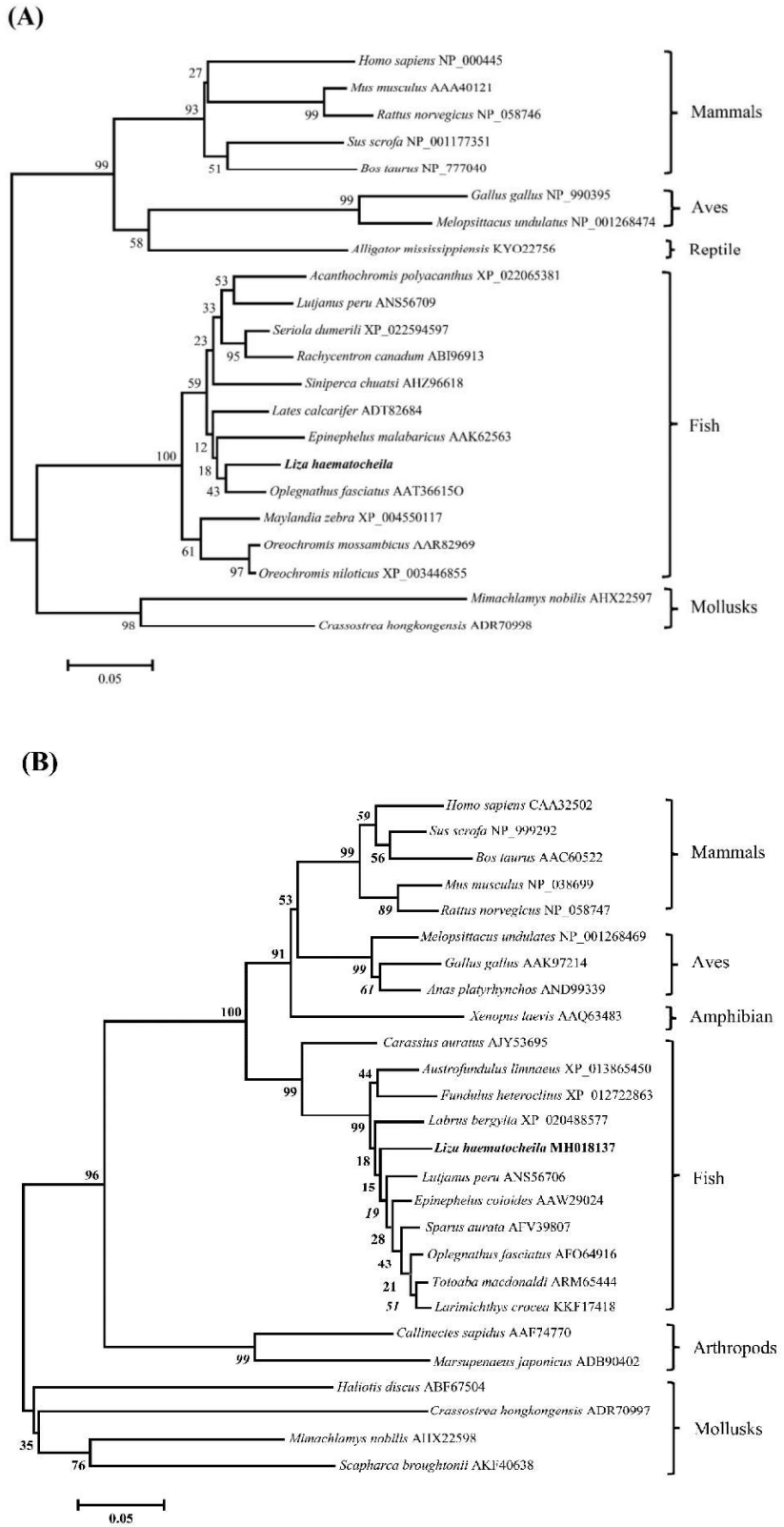
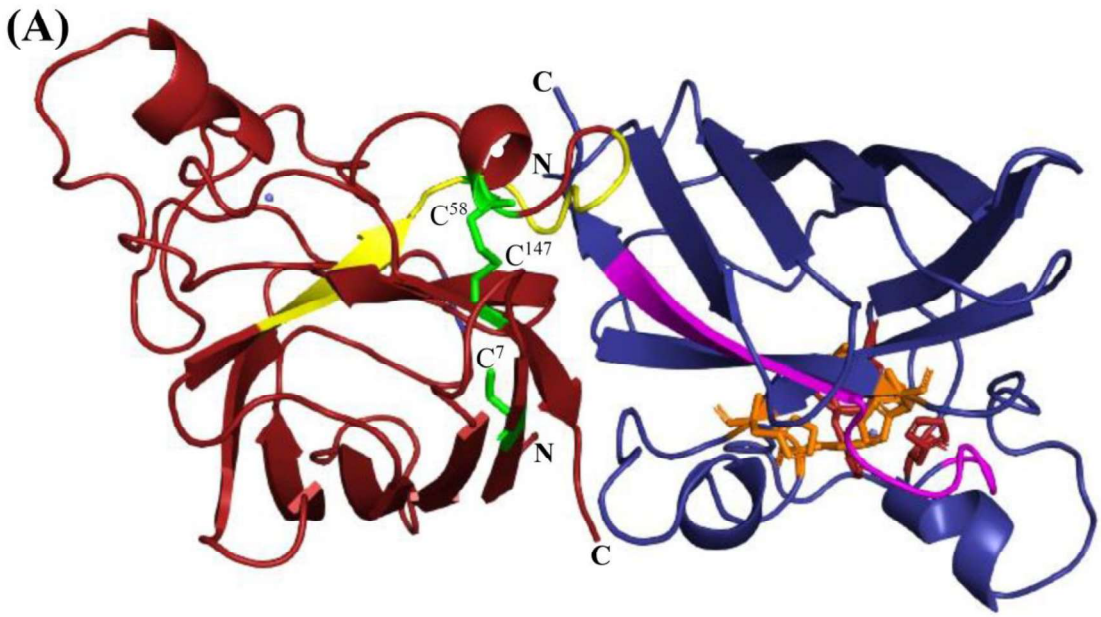


Figure 3. Phylogenetic trees of MuCuZnSOD (A) and MuMnSOD (B).
The tree structures were constructed using well-known protein sequences of CuZnSOD and MnSOD from different organisms. The bootstrap percentage after 5000 replication is indicated as a numerical value on each node. GenBank accession numbers are shown in front of each organism.

3.3 Characterization of the tertiary structure of MuCuZnSOD and MuMnSOD

The 3D structure of the MuCuZnSOD homo-dimer was identified by the template of crystal structure of human superoxide dismutase I (SMTL ID: 5yto.1). MuCuZnSOD showed 68.83 % sequence identity with the template whereas, MuMnSOD homo-tetramer was found by the template of the structure of human mitochondrial manganese superoxide dismutase (SMTL ID: 1n0j.1) with 84.34 % sequence identity (Figure 4). The tertiary structure of the MuCuZnSOD contained eight β-sheets and two short α-helical regions. MuMnSOD 3D view showed three β-sheets and eight α-helical structures. The cysteine residues presented in the MuCuZnSOD homodimer were responsible for the creation of disulphide bonds between the MuCuZnSOD monomers [95]. Moreover, the Cu^{2+} and Zn^{2+} ion binding sites of MuCuZnSOD were placed together to ensure the catalytic function, and Mn^{2+} binding sites in the MuMnSOD sequence also responsible for its activity.



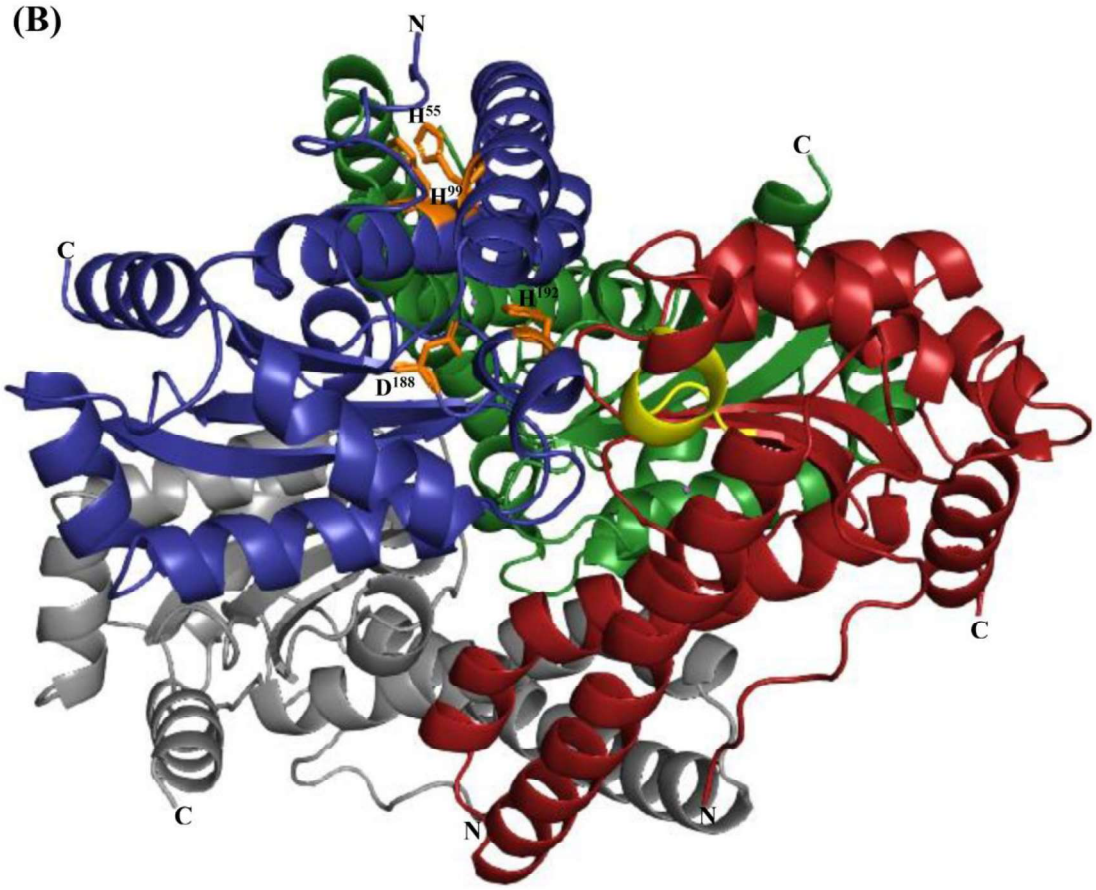


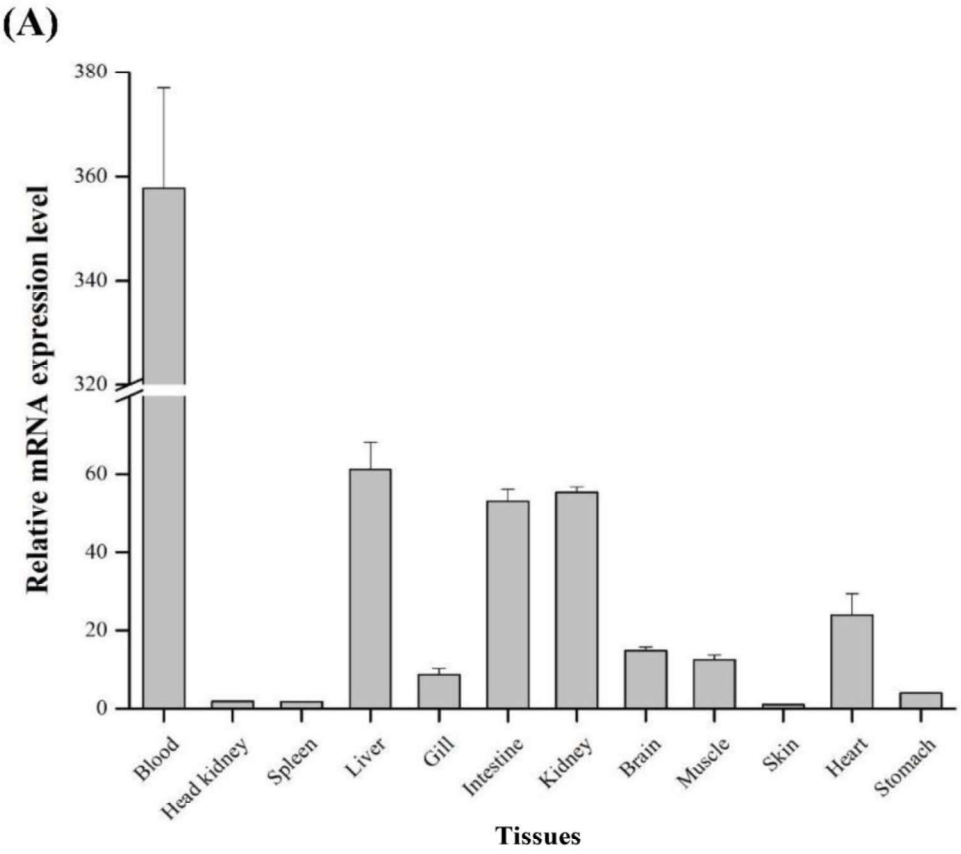
Figure 4. Tertiary structure of MuCuZnSOD (A) and MuMnSOD (B).
(A) MuCuZnSOD 3D structure. Two monomers of MuCuZnSOD was indicated in red and blue colour. The SOD_CU_ZN_1 signature is showed by yellow section and the SOD_CU_ZN_2 signature marked in pink. Green, red, and orange colour sticks represent the cysteine residues, Cu^{2+} binding sites and Zn^{2+} binding sites, respectively. (A) MuMnSOD 3D structure. Four monomers of MuMnSOD was displayed in red, blue, green and grey colour. Manganese and iron superoxide dismutase signature and Mn^{2+} binding sites were indicated in the yellow region and orange sticks.

3.4 Analysis of transcriptional tissue expression

The tissue expression profiles of *MuCuZnSOD* and *MuMnSOD* genes were analysed using total RNA from healthy mullet tissues of blood, head kidney, spleen, liver, gill, intestine, kidney, brain, muscle, skin, heart and stomach. The results showed that both *SOD* genes were expressed in all selected tissues, but the relative mRNA expression levels were varied on each tissue. However, highest mRNA expression

levels of MuCuZnSOD and MuMnSOD were indicated in the blood tissue, followed by the tissues of the liver, kidney and heart (Figure 5).

Blood is the main oxygen transporter in the whole body and it may be the main tissue which subjected to oxidative stress conditions due to direct exposure to the different ROS. Especially, hemocytes present in the invertebrates were responsible for phagocytosis against antigens in the blood as a defence mechanism [96]. Phagocytosis might be caused to induce oxidative stress by the ROS generation [97]. Therefore, both *SOD*s in the blood may be highly expressed to prevent the adverse effects of harmful ROS. Previous reports indicated that the *CuZnSOD* and *MnSOD* expression levels are highest in blood tissue as same as the current study findings[70,71,80,86].



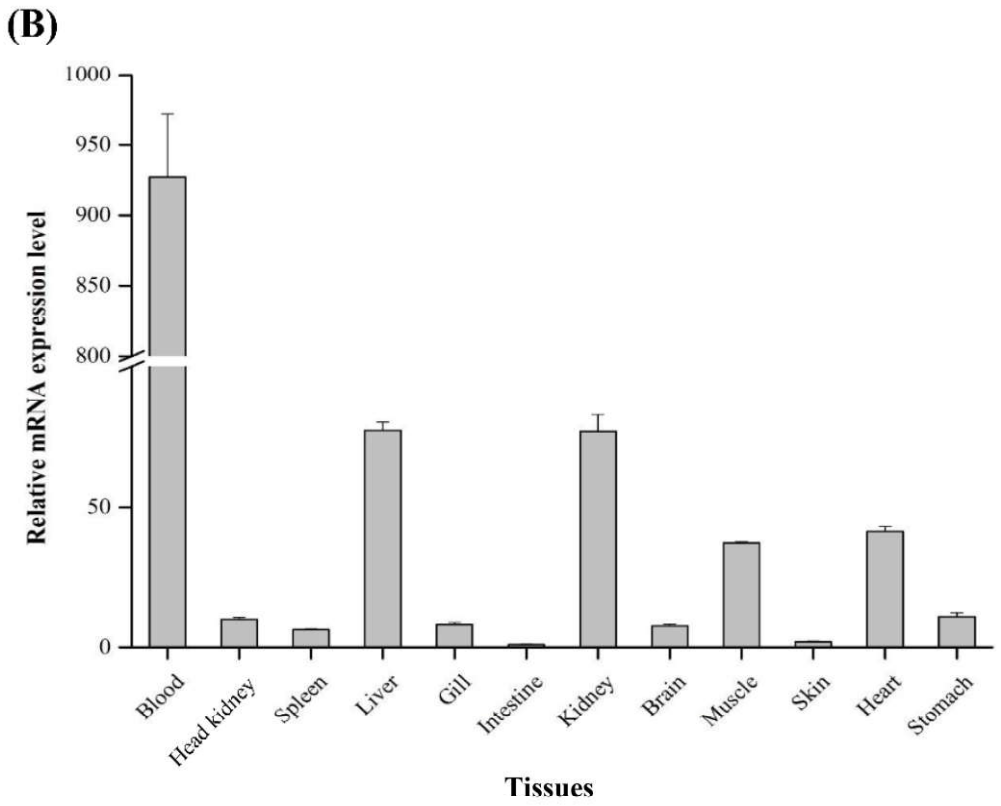


Figure 5. Tissue distribution of MuCuZnSOD (A) and (B) MuMnSOD.
Tissue distribution of *MuCuZnSOD* and *MuMnSOD*. The tissues were collected from healthy mullet fish, and expression levels in each tissue were analysed using real-time qPCR. All data are presented as mean values (n=3) and error bars show standard deviations (±SD).

However, previous studies showed that *MnSOD* expression was highest in the ovaries in *Hippocampus abdominalis* [94], gills in *Mytilus coruscus* [98] and heart in *Hemibarbus mylodon* [99], whereas *CuZnSOD* expression of *Ruditapes philippinarum* [100] and *Eriocheir sinensis* [101] was highest in gills and hepatopancreas, respectively. Moreover, tissues of liver, intestine, heart, brain and kidney show comparatively high *SOD* expression in mullet. It may be due to the high rate of metabolic activity and produce more energy than other tissues, causing the production of high intracellular ROS content. Both SODs may act as fundamental ROS scavengers throughout the whole aerobic metabolic process in the eukaryotic system [102].

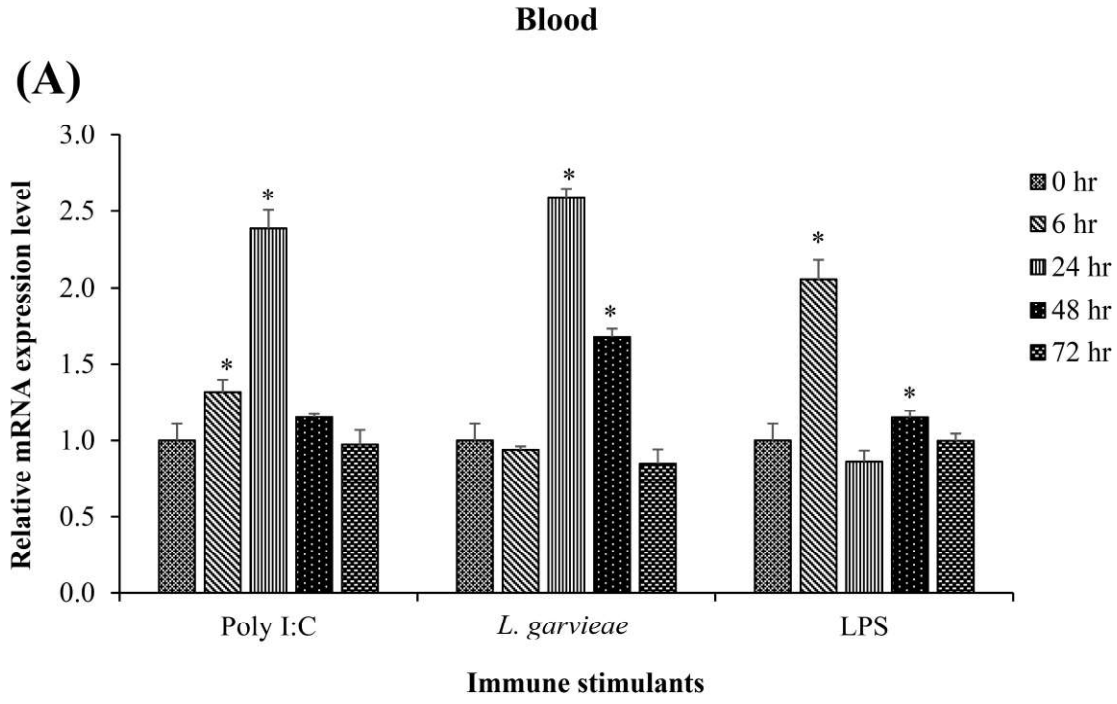
Therefore, these results suggest that *CuZnSOD* and *MnSOD* expression variations may be species-specific.

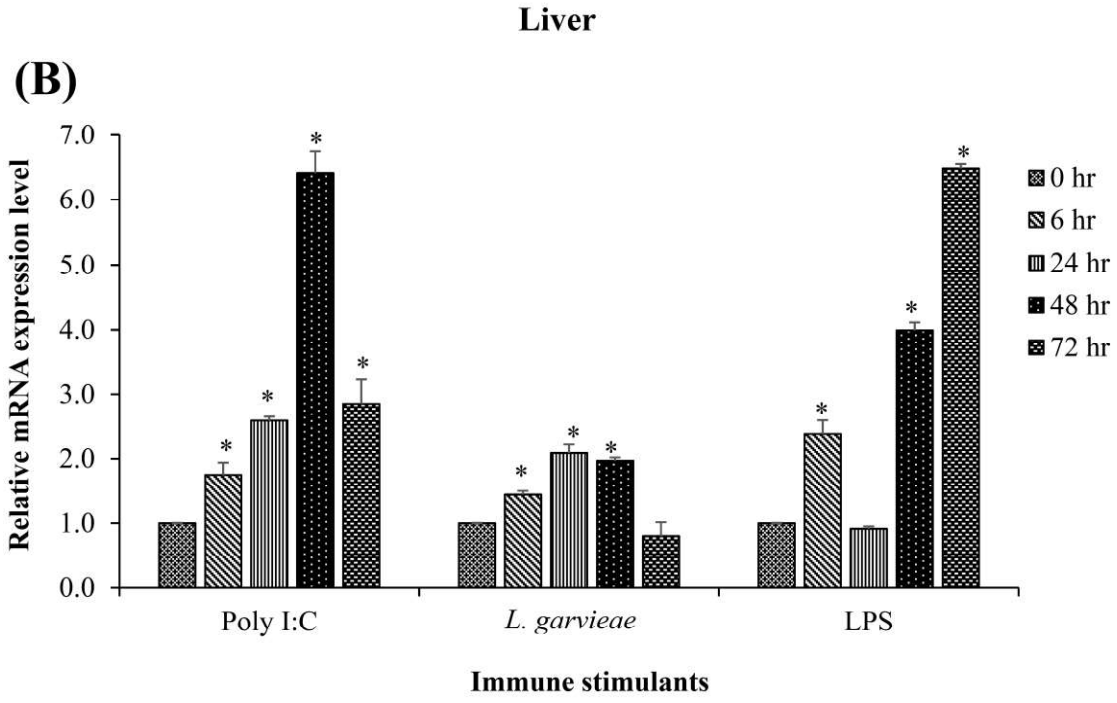
3.5 Temporal transcriptional expression profiles of MuCuZnSOD and MuMnSOD

Immune challenge experiment was performed to examine the immune function of *MuCuZnSOD* and *MuMnSOD* after injection of different immune stimulants and live bacteria in a time-dependent manner. Immune functions of *MuCuZnSOD* and *MuMnSOD* were investigated using blood and liver tissues (Figure 6).

The liver is the fundamental immune-related organ which is responsible for the innate immune system [103] and Periportal Kupffer cells presented in the liver are essential for nonspecific phagocytic activity [104]. Generation and accumulation of harmful ROS are occurred during phagocytic activity, resulting in considerable tissue damage [21]. Therefore, antioxidant enzymes might be associated with regulating the redox balance. Moreover, the blood is an important medium for innate immune functions in fish body [94].

Expression of *MuCuZnSOD* and *MuMnSOD* were significantly increased after injection of poly I:C at each time point in the liver. The highest expression of *MuCuZnSOD* can be observed at 48 h post injection in the liver and 24 h post-injection in blood. The *MuCuZnSOD* expression levels then declined at next time points in both tissues. The highest upregulation of *MuMnSOD* was seen at 48 h post injection of *Lactococcus garvieae*, and then the expression level declined at 72 h post injection in the liver. The mechanism of mRNA turnover might be the reason for the reduction of MuCuZnSOD and MuMnSOD expressions [108].





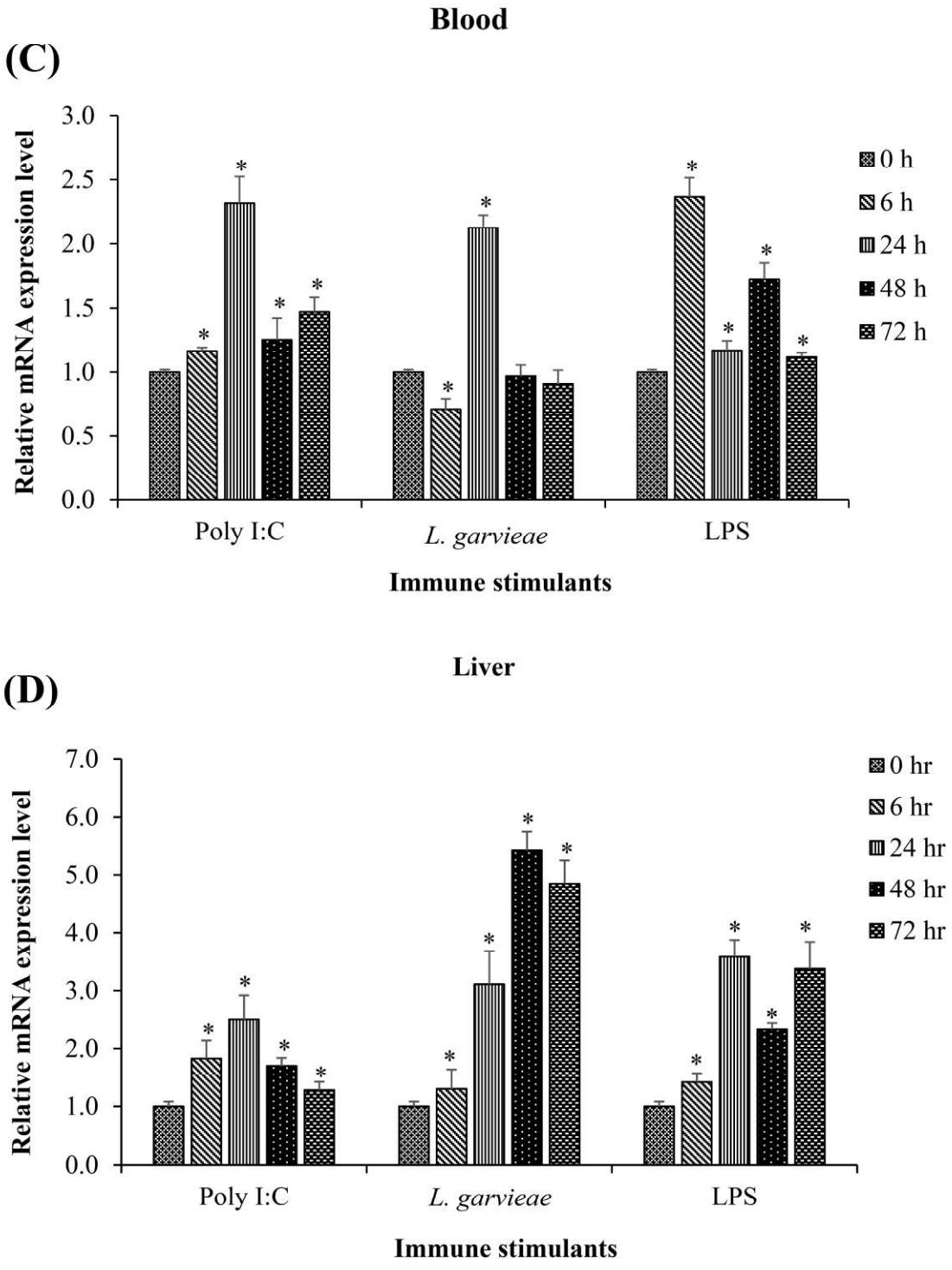


Figure 6. Temporal mRNA expression of *MuCuZnSOD* (A/B) and *MuMnSOD* (C/D) in blood and liver after immune challenge.
Polyinosinic: polycytidylic acid (Poly I:C:), *Lactococcus garvieae* (*L. garvieae*) and lipopolysaccharide (LPS) were used as immune stimulants. A significant difference between control and treatments are indicated by the * mark. All data are shown as mean values (n=3), and vertical bars represent the standard deviations (±SD).

In the blood cells, significant upregulation of *MuMnSOD* mRNA transcript was observed at 24 h post injection with poly I:C. The highest mRNA expression of *MuCuZnSOD* was indicated after *L. garvieae* injection at 24 h, and a similar expression pattern can be seen in both liver and blood tissues. *MuMnSOD* was highly expressed at 24 h and 48 h post-injection of *L. garvieae* in blood and liver, respectively. However, Results of *MuCuZnSOD* and *MuMnSOD* expression were varied with LPS treatment. The highest *MuCuZnSOD* and *MuMnSOD* expression with LPS were observed in liver at 72 h and 24 h post-injection, respectively, while the highest expression of both SODs was indicated at 6 h post injection in the blood. However, the expression patterns of *MuCuZnSOD* and *MuMnSOD* against different immune stimulants were different in liver and blood tissues. Hence, these results suggested that the expression of both *SOD*s may be tissue-specific with various immune stimulants in the immune challenge experiments.

Previous findings revealed that SODs are general stress responses that might be overexpressed by pathogens, heavy metals and harsh environmental conditions [62,98]. Pro-oxidant-antioxidant balance in host cells of mammals has been interfered due to induction of viral-mediated ROS [105]. The responsive modulation of *CuZnSOD* and *MnSOD* in seahorse and rock bream after poly I:C injection at late phase have been reported [70,80]. Moreover, expression of rockfish *CuZnSOD* and *MnSOD* levels were increased during the mid-phase of poly I:C treatment [71]. However, the challenge results of the present study suggested that viral-mediated ROS accumulation might be suppressed by *MuCuZnSOD* and *MuMnSOD* proteins.

L. garvieae is a potential virulent bacterium of fish species which involved in the development of infectious disease conditions including, streptococcosis and lactococcosis [106,107]. In Taiwan, *L. garvieae* was the main causative pathogen

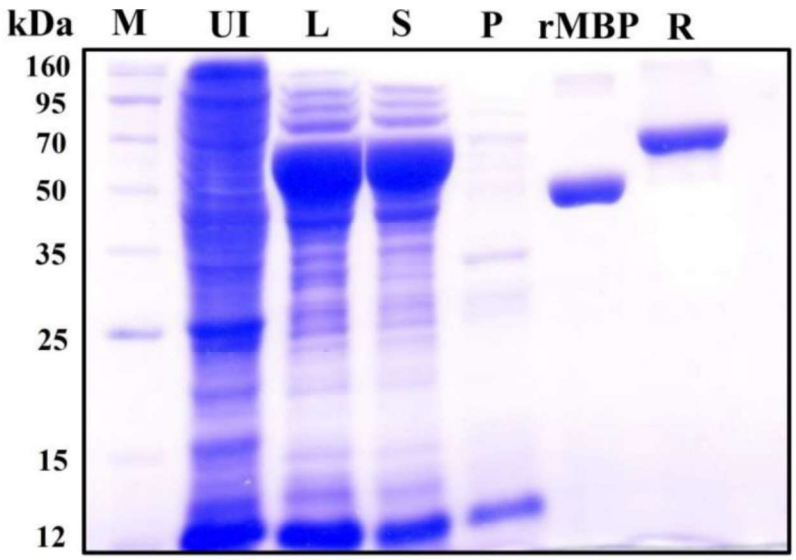
responsible for the high mortality of cultured grey mullet [108]. Moreover, the previous report showed that redlip mullets in cage culture facilities were affected by green liver disease outbreaks due to *L. garvieae* in Korea [11]. The *CuZnSOD* and *MnSOD* from seahorse and rockfish were significantly expressed after injection of live *Streptococcus iniae*, a gram-positive bacterium [70,71,94]. However, the *CuZnSOD* of freshwater prawn was significantly downregulated at 3 h time point upon the *L. garvieae* challenge [109]. Interestingly, these all findings indicated that the expression level of CuZnSOD might be species specific. According to these results, the present study suggested that *CuZnSOD* and *MnSOD* in mullet may be responsible for protection from the harmful ROS generated by bacteria.

LPS is an endotoxin present as a cell wall component in the gram-negative bacteria [110]. In mammalian cells, the *SOD* expression level has been increased due to endotoxin and oxidative stress conditions under the inflammatory process [111]. Challenge experiments conducted using seahorse [70,94], rockfish [71], manila clam [100] and bay scallop [112] showed that expression levels of both *SOD*s were elevated after injection of LPS stimulant. Secretion of tumor necrosis factor alpha (TNF-α) was stimulated by LPS and expression of *SOD* occurred through the activation of extracellular signal-regulated kinases 1 (ERK1) protein during the inflammatory process that has been reported earlier [49]. Therefore, the activity of SOD genes might be regulated by cytokines [113]. Collectively, the results obtained from the temporal mRNA expression analysis suggests that *MuCuZnSOD* and *MuMnSOD* may act as a great ROS scavenging agent under different stress conditions, specially created by pathogens.

3.6 Bacterial overexpression and recombinant protein purification

Recombinant proteins of MuCuZnSOD and MuMnSOD were overexpressed in ER2523 *E. coli* competent cells (NEB Express) and then purified. In protein purification, samples were obtained at different steps, and SDS-PAGE analysis was conducted. The size of the target protein band was shown in the cell lysate. The molecular mass of the purified rMuCuZnSOD and rMuMnSOD with the fusion protein (MBP) were approximately 58 kDa and 68 kDa, respectively (Figure 7). Therefore, present data have been indicated that both recombinant SOD expression and purification were successfully performed in the bacterial cells.

(A)



(B)

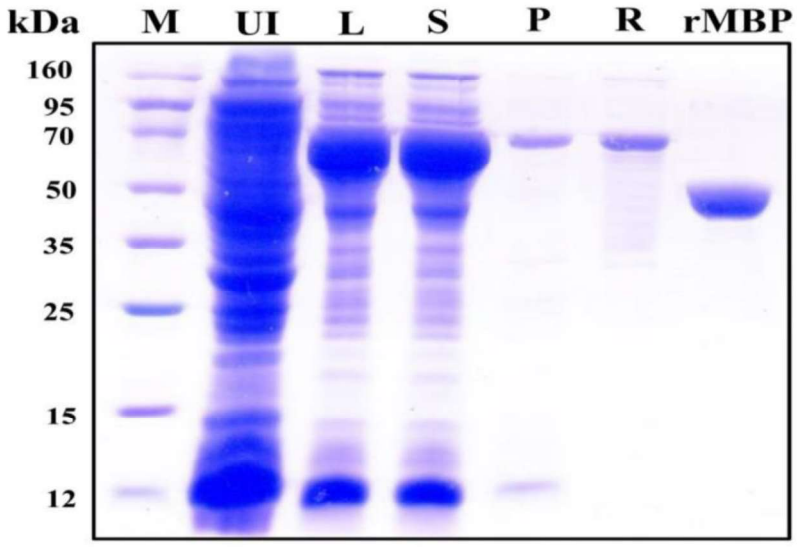


Figure 7. SDS-PAGE analysis of rMuCuZnSOD and rMuMnSOD in ER2523. (M) protein ladder; (UI) uninduced cellular extraction of *E. coli* ER2523; (L) protein induced cellular extraction; (S) soluble fraction after bacterial cell lysis; (P) insoluble content after centrifugation of bacterial cell lysis; (rMBP) recombinant maltose binding protein; (R) purified rMuCuZnSOD fusion protein.

3.7 Analysis of the antioxidant functions of recombinant SODs

Superoxide radicals formed during oxidative stress conditions in the intracellular compartment and extracellular matrix are effectively removed by both enzymes from the dismutation mechanism [114,115]. Therefore, the antioxidant assay was performed using both recombinant SODs and assessed the antioxidant functions of MuCuZnSOD and MuMnSOD in different conditions. The superoxide scavenging ability of MuCuZnSOD and MuMnSOD were evaluated using conventional XOD assay with a chromogenic indicator. The reaction of xanthine and xanthine oxidase produce the uric acid and H_2O_2, and the main by-product is the superoxide molecules. The superoxide anions are important to convert the NBT into NBT-diformasan; hence, the colour change is occurred due to NBT conversion, and it is measured by spectrophotometer at a 560 nm. The NBT-diformasan level is reduced by the

conversion of superoxide into H_2O_2 that could be calculated as a relative SOD activity of MuCuZnSOD and MuMnSOD [80,94].

3.7.1 Determination of antioxidant ability of rMuCuZnSOD and rMuMnSOD at different pH values

Conventional XOD assay was conducted in the different pH values, including pH 3 to pH 11 and the rMuCuZnSOD and rMuMnSOD activities were highest in pH 9 and pH 7, respectively (Figure 8).Therefore, effect of other conditions on MuCuZnSOD activity and MuMnSOD activity were observed under the selected pH levels. Results obtained from the present experiment indicated that the acidic pH levels were caused to inhibit the SOD activity. The CuZnSOD activity depends on alkaline pH that shows the main function of charged residues such as lysine [116]. At alkaline pH levels, the dismutation rate is accelerated due to the reduction of positive charges on lysine residues, and it regulates the presence of the active sites in the enzyme [117]. The superoxide radicals generate at the neutral pH cause for electrostatic repulsion among negatively charged molecules that leads to decrease in the dismutation process [118]. At acidic pH, imidazole rings of the manganese ion ligand His residues might be protonated. Therefore, the metal-ligand interactions interfere in the low pH levels [113]. However, purified ark shell MnSOD activity was high at acidic pH values [74]. However, most of the previous studies indicated that SOD activity of seahorse, rockfish and rock bream were highest at alkaline pH levels [70,71,80]. Altogether, these research finding revealed that SODs are responsible for protecting the host organism at a wide range of pH levels under the oxidative stress.

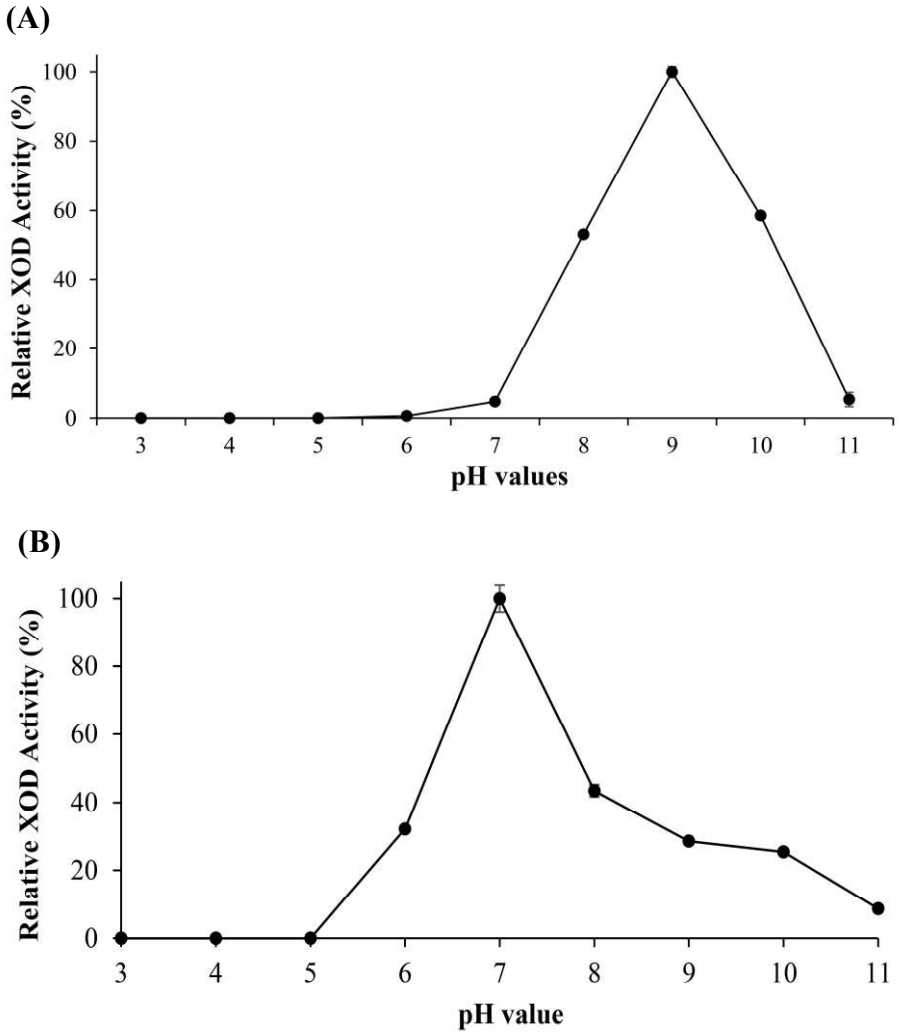


Figure 8. Determination of optimum pH levels for the activity of rMuCuZnSOD and rMuMnSOD using xanthine oxidase assay.

3.7.2 Determination of the antioxidant activity of rMuCuZnSOD and rMuMnSOD at different temperatures

The temperature range of 0-85 °C was selected to perform the XOD assay (Figure 9). The highest rMuCuZnSOD activity and rMuMnSOD activity were observed at 25 °C and the significant SOD activity was distributed at temperature range (5-65 °C). Therefore, the rMuCuZnSOD and rMuMnSOD proteins may be identified as stable enzymes under different temperatures. Previous findings revealed that the optimum temperature for SOD activity was 25 °C. Therefore, these findings support the current

findings related to the optimum temperature [70,71,80,94,119]. In ark shell, 50 °C was the optimum temperature for its MnSOD activity [74], while the rock bream MnSOD activity was highest at 20 °C [86]. Moreover, manila clam and bay scallop CuZnSOD activity was highest at 20 °C [100,112]. Therefore, these findings suggest that CuZnSOD and MnSOD play a significant role against hazardous ROS in different aquatic species at different thermal conditions.

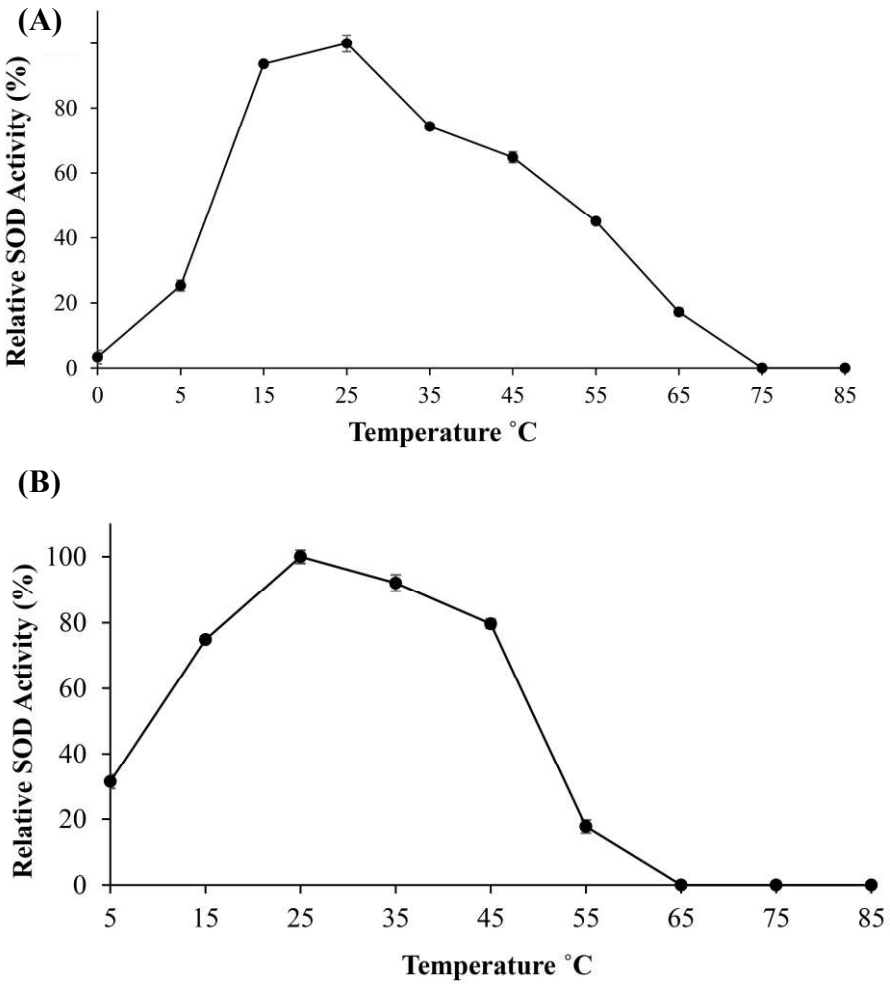
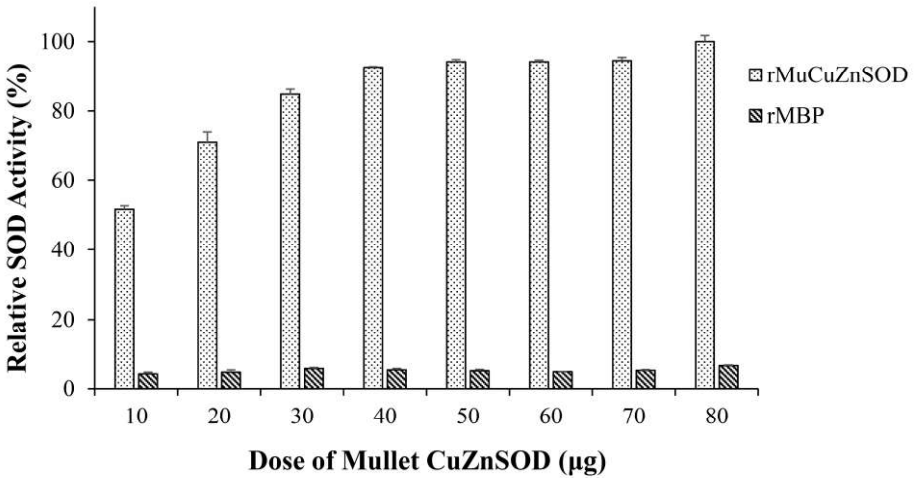


Figure 9. Determination of optimum temperature for the activity of rMuCuZnSOD and rMuMnSOD using xanthine oxidase assay.

3.7.3 Determination of effect of enzyme dose on antioxidant activity of rMuCuZnSOD and rMuMnSOD

The relative SOD activity of rMuCuZnSOD and rMuMnSOD were measured using different dosages of recombinant protein (10-80 μg) (Figure 10) Relative antioxidant activity of SODs was significantly increased with the increasing of recombinant protein dosage. Recombinant MBP did not indicate the significant difference in relative antioxidant activity with the dosage. Therefore, it has been confirmed that the MBP is only a fusion protein that does not have a superoxide scavenging ability. However, significant ROS scavenging activity was observed at lower doses (10-20 μg) of recombinant proteins. Previous findings also confirmed that lower dosages of recombinant SOD were responsible for significant superoxide scavenging activity and higher dosages could increase the relative antioxidant function up to its maximum level [70,71,80,94]. Takentogether, these results showed that lower doses of recombinant SOD enzymes might be required for the superoxide scavenging activity in mullet.

(A)



(B)

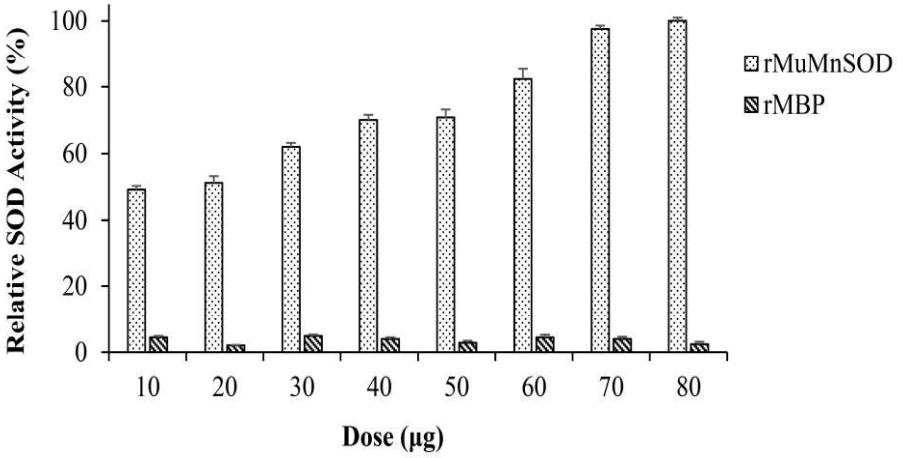


Figure 10. Conventional xanthine oxidase (XOD) assay for the determination of the effect of rMuCuZnSOD dose (µg) at optimum conditions.

3.7.4 Determination of the effect of inhibitory factors on rMuCuZnSOD and rMuMnSOD

The effect of inhibitors on SOD activity was evaluated using different inhibitory solutions, including ethylene EDTA, NaN_3, DDC and KCN. The NaN_3, DDC and KCN significantly inhibited the antioxidant activity of rMuCuZnSOD, while the inhibition of rMuMnSOD activity is not significant under the treatment of EDTA and NaN_3. However, KCN and DDC indicated very strong inhibitory capacity (Figure 11). Several previous results related to the effect of inhibitors on SODs were agreed with the current results [70,71,120,121]. It has been revealed that the superoxide scavenging activity of SOD was not inhibited by EDTA [120]. The SOD activity was selectively inhibited by millimolar concentrations of DDC and KCN [122]. The active sites of SODs were competitively invaded by cyanide ion (CN^-). Hence superoxide anion binding ability was suppressed. Furthermore, copper and manganese ions were also specifically chelated by DDC chemical [123]. Therefore, these results suggested that the antioxidant

activity of both SODs might be directly interrupted by KCN and DDC like inhibitory factors.

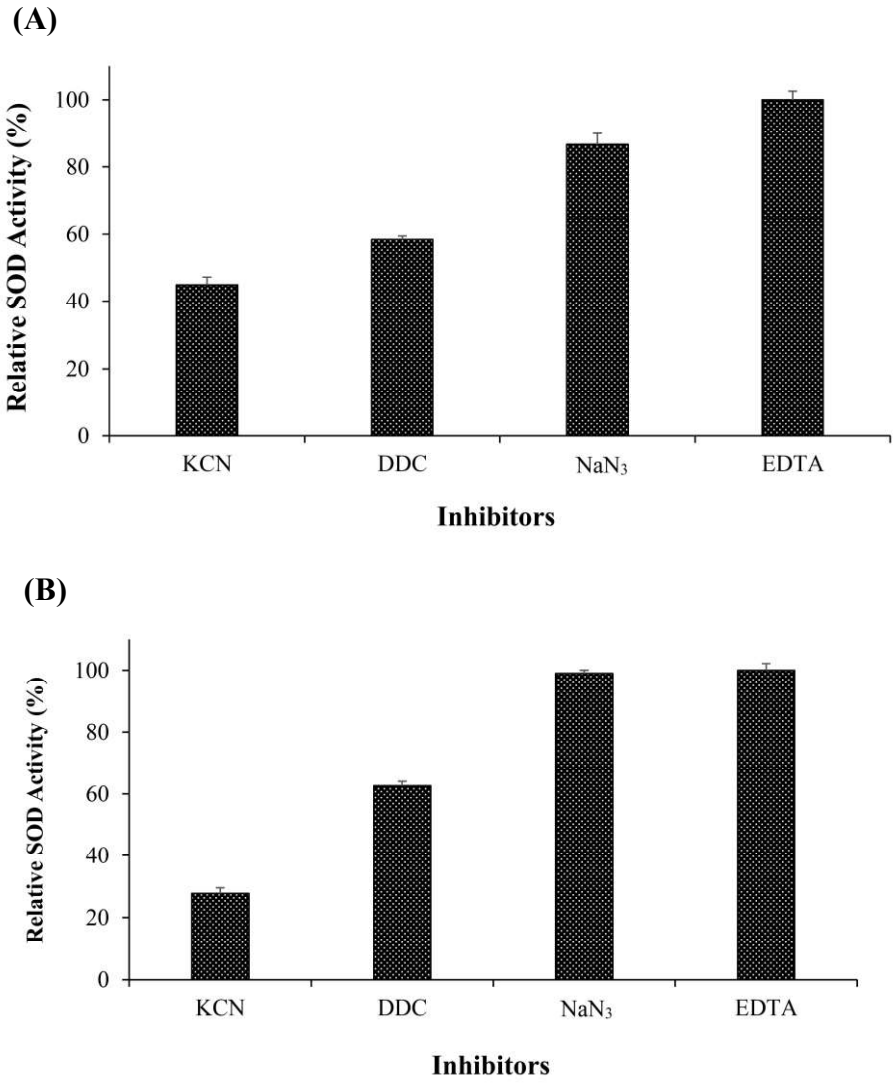


Figure 11. Determination of the effect of inhibitors for the activity of rMuCuZnSOD and rMuMnSOD using xanthine oxidase assay.

3.8 Determination of Peroxidation activity of rMuCuZnSOD

The peroxidation activity of rMuCuZnSOD was evaluated by measuring the cell survival rate (MTT assay) under the condition of cytotoxicity. The presence of HCO_3^- and rMuCuZnSOD impacted on increasing the number of viable cells. Moreover, the cell survival rate was gradually enhanced with increasing of the concentration of

rMuCuZnSOD protein. Highest cell survival rate was identified after treatment of 100 µg/mL of rMuCuZnSOD and viable cell count was not significantly impacted by rMBP (Figure 12).

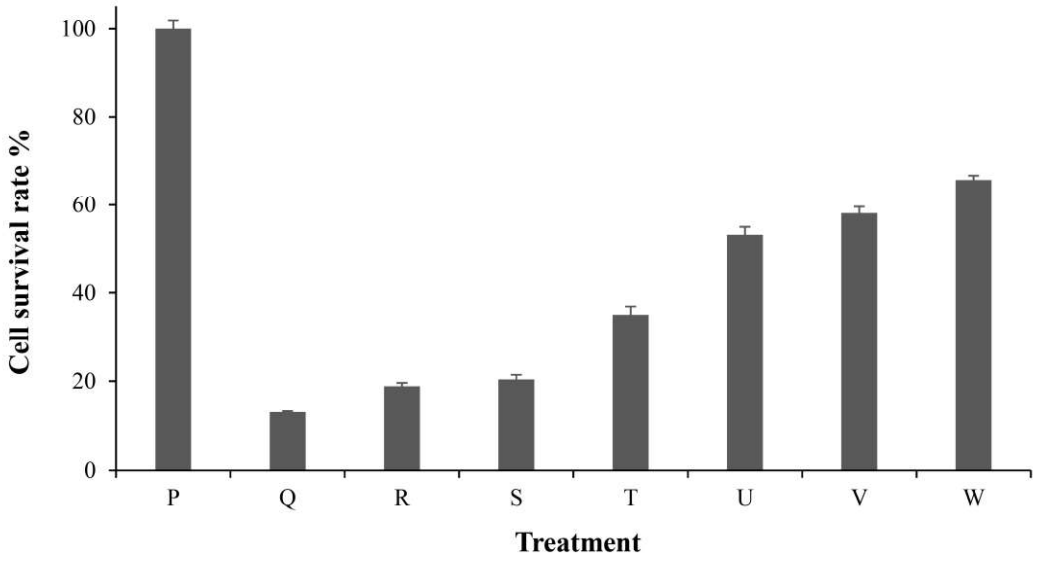


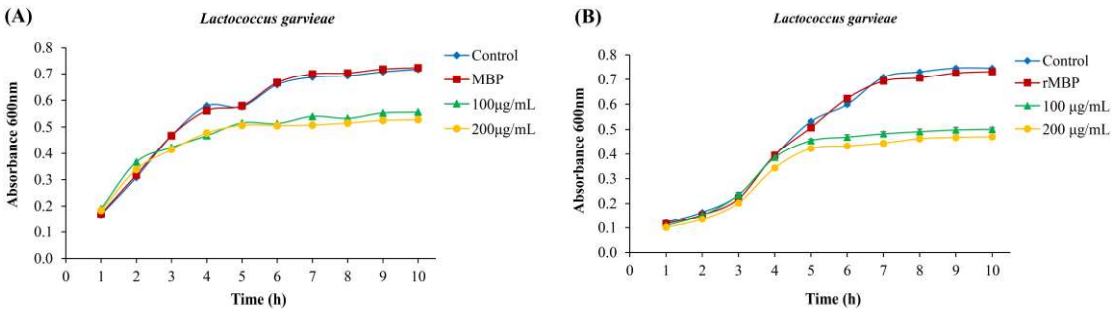
Figure 12. Effect of rMuCuZnSOD on cell survival rate after treatment of H_2O_2. The viable cell count was detected by the MTT assay. All data are shown as mean values (n=3) and vertical bars represent the standard deviations (±SD). Treatments are indicated as follows: (P) control sample, (Q) 400 µmol H_2O_2, (R) 20 mM $NaHCO_3$ + 400 µmol H_2O_2, (S) 100 µg/mL rMBP + 20 mM $NaHCO_3$ + 400 µmol of H_2O_2, (T) 25 µg/mL rMuCuZnSOD + 20 mM $NaHCO_3$ + 400 µmol H_2O_2, (U) 50 µg/mL rMuCuZnSOD + 20 mM $NaHCO_3$ + 400 µmol H_2O_2, (V) 75 µg/mL rMuCuZnSOD + 20 mM $NaHCO_3$ + 400 µmol of H_2O_2 and (W) 100 µg/mL rMuCuZnSOD + 20 mM $NaHCO_3$ + 400 µmol of H_2O_2.

Previous findings indicated that CuZnSOD has a capacity to breakdown the peroxide molecules in the presence of HCO_3^- ions rather than its primary anti-oxidant function [124,125]. The present study demonstrated that the rMuCuZnSOD protein has displayed a significant peroxidation activity by increasing the viable cell count against the oxidative stress generated by H_2O_2. The CuZnSOD activity might be suppressed by blocking the Cu^{+2} ion binding sites due to high H_2O_2 concentration [126]. Nevertheless, it has been revealed that CuZnSOD leads to protect the cells against toxicity created by H_2O_2 and degrade the H_2O_2 in the presence of HCO_3^- ions in the medium [124,127].

Furthermore, the redox cleavage of H_2O_2 might be activated by the HCO_3^- ions binding with the Arg^{144} residue presented in the MuCuZnSOD [125]. Results obtained from the MTT assay revealed that the MuCuZnSOD contains a significant peroxidation activity in the presence of HCO_3^- ions in the medium which is caused for enhancing the cell survival rate of H_2O_2 treated Vero cells.

3.9 Determination of the antibacterial activity of rMuCuZnSOD and rMuMnSOD

Antibacterial activity of both SODs were determined by evaluating the growth rates of specific bacteria with the incubation of rMuCuZnSOD protein and rMuCuZnSOD protein (Figure 13). Especially, growth reduction of *M. luteus*, *L. garvieae* (gram-positive) and *E. coli* (gram-negative) were compared with the MBP and PBS control samples. All three bacterial strains did not show significant growth difference with control groups at the early phase, but after 4-6 h treatment of recombinant SODs, they showed a limited growth and significantly affected the *L. garvieae* growth under MuMnSOD treatment. Furthermore, the temporal mRNA expression of *MuCuZnSOD* and *MuMnSOD* were elevated after 6 h post injection of *L. garvieae* and the antibacterial function against *L. garvieae* was clearly shown after 4 h to the 5 h treatment of recombinant proteins.



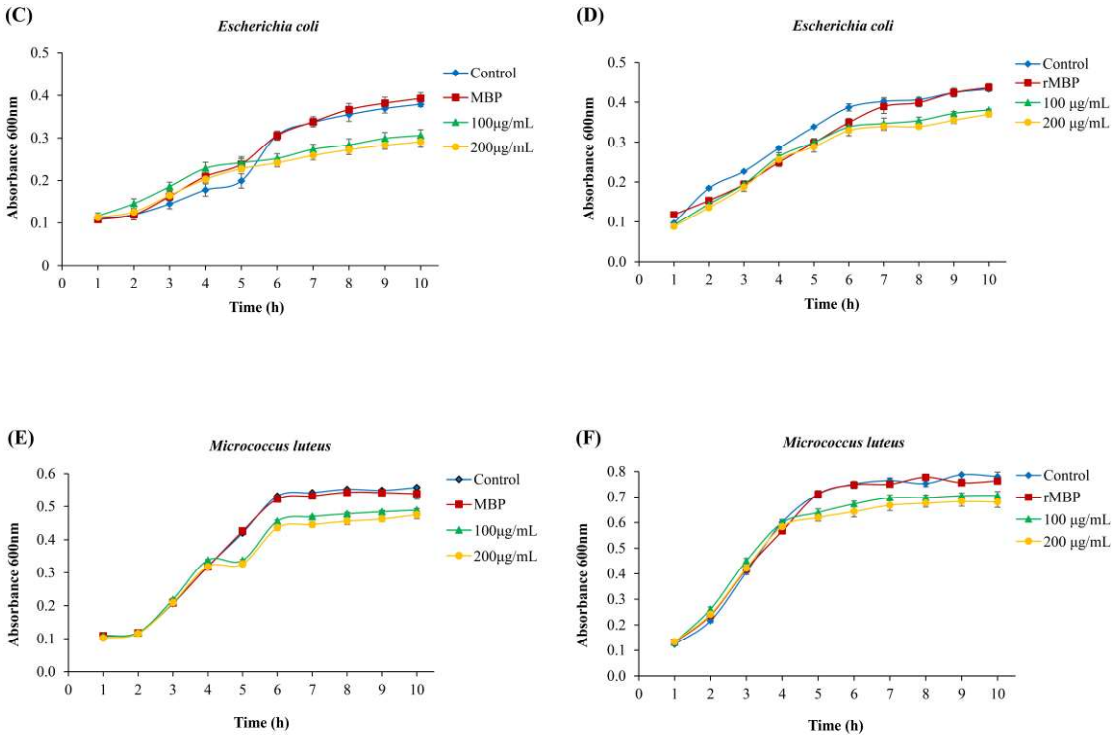


Figure 13. The growth rate of *L. garvieae*, *E. coli*, and *M. luteus* after treating with recombinant MuCuZnSOD (A/C/E) and MuMnSOD (B/D/F) proteins.
All data are indicated as mean value (n=3).

These results suggested that both proteins may perform a defense mechanism against the *L. garvieae* infection. Moreover, results obtained from the antibacterial assay showed that both gram-positive and gram-negative bacterial growth was decreased after treatment of recombinant SODs. This growth retardation may be occurred due to the activity of specific active sites present in the amino acid sequences of SODs. An earlier study reported that recombinant MnSOD of *Scapharca broughtonii* also has indicated an antibacterial function against both gram-positive and gram-negative bacteria [74]. LPS binding function of *Crassostrea gigas* SOD has been previously reported. It may have an ability to recognize the bacterial cells by opsonic function and avoid the bacteria growth through adhering to the trans-membrane receptors (integrin) of hemocytes. It has been shown that *C. gigas* SOD contains a specific LPS binding motif recognized in the endotoxin receptor called CD14, which

indicated an affinity to the LPS and the *E. coli* strains [128]. Although limited studies were reported under the antibacterial mechanism of SODs, the current study clearly shows that CuZnSOD and MnSOD from mullet possess a capacity to combat against the bacterial attack and could be introduced as immune-related genes.

4 Conclusion

In brief, the current study exhibits the structural and functional characteristics of CuZnSOD and MnSOD from mullet fish. Bioinformatics analysis showed that both SODs were well conserved among the other analysed orthologs. The relative mRNA expression profile of both SODs indicated that blood is the highest responsive tissue. The challenge expression study demonstrated synchronized modulation of SODs and under different pathogenic stimulants, revealing its involvement in the mullet immune system. The conventional xanthine oxidase assay has illustrated the antioxidant ability of recombinant SODs. In addition, the peroxidation activity of rMuCuZnSOD in the presence of HCO_3^- ions was confirmed using the MTT assay. Furthermore, it has been highlighted the antibacterial function of both recombinant proteins with the three different bacteria. These findings collectively suggest that MuCuZnSOD and MuMnSOD may perform crucial roles in antioxidant defence system and immune system in the mullet fish.

5 References

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