# FUNCTIONAL ANALYSIS OF GLUTATHIONE S-TRANSFERASE FROM THE EXTREMOPHILE *Halothece* sp. PCC7418

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การวิเคราะห์เชิงหน้าที่ของกลูตาไธโอน เอส-ทรานส์เฟอเรสจากเอ็กซ์ทรีโมไฟล์ *Halothece* sp. PCC7418

นายชนาญวัต กอธีระกุล



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ชนาญวัต กอธีระกุล : การวิเคราะห์เชิงหน้าที่ของกลูตาไธโอน เอส-ทรานส์เฟอเรสจากเอ็กซ์ทรีโมไฟล์ *Halothece* sp. PCC7418. ( FUNCTIONAL ANALYSIS OF GLUTATHIONE S-TRANSFERASE FROM THE EXTREMOPHILE *Halothece* sp. PCC7418) อ.ที่ปรึกษาหลัก : รศ. ดร.รุ่งอรุณ วาดิถี สิริศรัทธา

กลูตาไธโอน เอส-ทรานส์เฟอเรส (GST) เป็นกลุ่มของเอนไซม์ที่มีหน้าที่หลากหลายและถูกกำหนดรหัสจากกลุ่มยีนขนาดใหญ่ การศึกษาในอดีตพบว่า GST มีบทบาทสำคัญในกระบวนการกำจัดสารพิษระดับเซลล์ การควบคุมสมดุลรีด็อกซ์ และการตอบสนองต่อภาวะเครียดต่าง ๆ ซึ่งถึงแม้ว่าจะมีการศึกษากลุ่มของ GST ในหลายสิ่งมีชีวิต แต่บทบาทของยีน GST ในสิ่งมีชีวิตกลุ่มออโตโทรปส์ชั้นต่ำที่ใช้ออกซิเจน เช่น ไซยาโนแบคทีเรีย ยังไม่มีการศึกษาที่แพร่หลายในปัจจุบัน ในวิทยานิพนธ์นี้จึงได้ทำการศึกษาและวิเคราะห์เชิงหน้าที่ของ GST จากไซยาโนแบคทีเรีย *Halothece* sp. PCC7418 (หลังจากนี้จะเรียกว่า *Halothece* GSTs) จากการสืบค้นข้อมูลชีวสารสนเทศพบว่ายีนกำหนดรหัส GST ในไซยาโนแบคทีเรียดังกล่าว ประกอบด้วย GST_0647, GST_0729, GST_1478 และ GST_3557 ทั้งสี่ยีนมีความหลากหลายของสายวิวัฒนาการ และจัดอยู่ในกลุ่มที่ต่างกัน โดย GST_0647 และ GST_1478 เป็นยีนที่ยังมีความคล้ายคลึงกัน แต่ GST_0729 และ GST_3557 นั้นแตกต่างออกไปอย่างสิ้นเชิง จากนั้น *Halothece* GSTs ได้ถูกโคลนและแสดงออกในเซลล์ *E. coli* BL21 ซึ่งผลการวิเคราะห์การทนต่อภาวะเครียดพบว่า เซลล์ที่แสดงออก GST_3557 มีอัตราการรอดชีวิตหลังเลี้ยงภายใต้ภาวะเครียดจากเกลือ และภาวะเครียดแบบออกซิเดทีฟ มากกว่าเซลล์อื่น ๆ อย่างมีนัยสำคัญ โดยจำนวนเซลล์ GST_3557 ที่รอดชีวิตจากภาวะเครียดจากเกลือมีจำนวนมากกว่าชุดควบคุม empty vector ถึง 18 เท่า แสดงให้เห็นถึงบทบาทของ GST_3557 ที่สำคัญมากต่อการอยู่รอดของเซลล์ที่แสดงออก ภายใต้ภาวะเครียดจากปัจจัยที่ไม่มีชีวิต ในการวิเคราะห์เชิงหน้าที่ของ GST_3557 พบว่า GST_3557 มีแอคติวิตีของ GST โดยใช้ กลูตาไธโอน (GSH) และ 1-chloro-2, 4- dinitrobenzene (CDNB) เป็นสารตั้งต้น ในช่วง pH หลากหลาย ตั้งแต่ 6.5 ถึง 10.5 โดยค่าทางจลศาสตร์ของเอนไซม์ ได้แก่ $K_m$ ต่อ CDNB และ GSH มีค่า 0.14±0.02 และ 0.74±0.29 mM ตามลำดับ แสดงให้เห็นว่า GST_3557 สามารถจับกับสารประกอบ CDNB และเร่งปฏิกิริยาได้อย่างจำเพาะมากกว่า อย่างไรก็ตามพบว่า GST_3557 ไม่แสดงแอคติวิตีของเพอร์ออกซิเดส ผลการทดลองนี้ทำให้สามารถเห็นภาพกลไกระดับเซลล์และระดับโมเลกุลของ GST จากไซยาโนแบคทีเรีย ซึ่งเป็นหนึ่งในเอนไซม์ที่มีความสำคัญอย่างยิ่งในการตอบสนองต่อภาวะเครียด และการเจริญเติบโตภายใต้สิ่งแวดล้อมที่ไม่เหมาะสม รวมถึงทำให้เข้าใจกลไกการตอบสนองทางสรีรวิทยาของเซลล์ภายใต้ระบบการแสดงออกที่ต่างกันได้มากขึ้น

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| สาขาวิชา | จุลชีววิทยาและเทคโนโลยีจุลินทรีย์ | ลายมือชื่อนิสิต ..... |
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KEYWORD: Glutathione s-transferase, Extremophile, Cyanobacteria, Salt Stress, Oxidative Stress

Chananwat Kortheerakul : FUNCTIONAL ANALYSIS OF GLUTATHIONE S-TRANSFERASE FROM THE EXTREMOPHILE *Halothece* sp. PCC7418. Advisor: Assoc. Prof. Rungaroon Waditee-Sirisattha, Ph.D.

Glutathione S-transferase (GST) are a set of multifunctional enzymes encoded by large gene families. It has been functionally demonstrated that GSTs play vital roles in cellular detoxification, regulation of redox-dependent process, and stress responses. Although the GST gene family has been extensively studied across taxa, the function of the GST genes in primordial oxygenic phototrophs such as cyanobacteria is poorly understood. In this thesis, GSTs from extremophilic cyanobacterium *Halothece* sp. PCC7418 (hereafter *Halothece* GSTs) were identified and functionally characterized. The genome-based analysis showed that there were four GSTs in *Halothece* 7418 (GST_0647, GST _0729, GST _1478, and GST _3557). Phylogenetic relationship revealed that these cyanobacterial GSTs were highly divergent. GST _0647 and GST _1478 are paralogous genes while other two GSTs (GST_0729 and GST _3557) are distinct. These *Halothece* GSTs were cloned and successfully expressed in *E. coli* BL21. Stress tolerance of expressing cells were evaluated under salt and oxidative stresses. Amongst four expressing cells, the GST _3557 performed the greatest tolerance to oxidative and salt stresses. Viable cell count of GST_3557 under salt stress was higher than empty vector control approximately 18 folds. These results support the protective role and vital function of GST_3557 against abiotic stress in a heterologous expression system. Recombinant GST_3557 exhibited GST activity toward 1-chloro-2, 4-dinitrobenzene (CDNB) and glutathione (GSH) with a broad range of activity at pH 6.5–10.5. Kinetic parameters showed the apparent $K_m$ for CDNB and GSH was 0.14±0.02 and 0.74±0.29 mM, respectively. Thus, GST _3557 had high affinity for electrophilic substrate, CDNB. In case of peroxidase activity, GST _3557 did not perform activity in all our conditions tested. Results from this study provided insight into the molecular and cellular functions of cyanobacterial GST, which is less understood compared to other counterparts. These results contribute toward understanding the mechanism behind physiological plasticity under a heterologous expression system.



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# CHAPTER I

# INTRODUCTION

Cyanobacteria, an enormously diverse group of prokaryotes, are oxygenic phototrophs that ubiquitously inhabit our planet. They play vital roles as primary producers in ecosystem, serving as human foods and sources of ingredients, as well as being a tool for industrial biotechnology (Frigaard, 2018). Some species should be noted as toxin producer and cause environmental issues, such as algae blooms (Puschner & Moore, 2013). Through deep time evolution, cyanobacteria are extremely adaptive and have developed unique survival strategies. Thus, various species can thrive under extreme environments encompassing vastly diverse terrains (Bolhuis et al., 2014; Hagemann, 2011; Thomas et al., 2005). A number of cyanobacteria are regarded as extremophiles. They inhabit and thrive in one or more extremely environmental conditions, ranging from high temperature (thermophiles), high salinity (halophiles), strong acidic or basic pH (acidophiles or alkaliphiles) and high pressure (barophiles) (Rathinam & Sani, 2018). The characteristics of extremophilic cyanobacteria that allow them to overcome adverse conditions by having intrinsic characteristics of adaptive or stress-responsive proteins make them useful models for the study of enzymology. Especially, the extremophilic cyanobacteria possessing distinct enzymes, the so-called extremozymes. These would be applied in several approaches, such as bioremediation, metabolic engineering, medical biotechnology, agricultural biotechnology and industrial bioprocess (Elleuche et al., 2014).

The ability to survive under extreme condition is another reason making the extremophile as a useful model for the study of molecular, cellular and physiological stress responses. Extremophiles inhabit under fluctuated stress environments, so cellular detoxification and stress responses are of the key mechanisms against molecular and cellular stresses. Heavy metals and xenobiotics are stress factors polluted in the environments. High concentration of heavy metals or xenobiotics are known as toxicants to cells by disruption of metabolism and/or cellular component damages. However, some cyanobacteria are capable of resisting

these harsh environments. For instance, *Nostoc muscorum* produces protein on cell surface and utilizes to bind with heavy metal cations, such as Pb(II) and Cu(II). Thereafter, heavy metals slowly take up into cells and turn to less toxic form, together with detoxification processes (Hazarika et al., 2015). *Prochlorococcus* sp. is another example of metal-tolerant microbes. This cyanobacterium possesses unique efflux pump to remove excess metal ion absorbed into cells, to maintain ionic balance and cellular homeostasis (Saunders & Rocap, 2016). Salt stress is another stress factor with the most significant environmental problems facing the world (Waditee et al., 2005). High salt concentrations interfere cellular ionic balance and cause the accumulation of misfolded and unfolded proteins *in vivo*. Salt stress coincident with osmotic stress and ion toxicity can also lead to cell death (Wang et al., 2011). In addition, both salt stress and heavy metal can trigger the formation of intracellular Reactive Oxygen Species (ROS). ROS are highly reactive molecules that cause structural biomolecule (e.g. carbohydrates, nucleic acids, lipids, and proteins) damages, and alteration of their functions. They are both endogenously generated from cellular metabolism and converted from xenobiotic substrates that directly taken up from environments. These substrates can be either hydro-peroxide compounds, such as hydrogen peroxide ($H_2O_2$) and cumene hydroperoxide (CuOOH), or metal ions, including iron, copper, cadmium, mercury, nickel, lead, and arsenic. ROS also mainly cause the lipid oxidation and react with nuclear proteins and DNA, which lead to cellular mortality (Birben et al., 2012). Thus, the accumulation of ROS causes the defective mechanisms and finally resulted in cellular oxidative stress. To survive under harsh environment that always induces cellular stresses, detoxification is the essential tool developing to deal with these stress factors.

Cellular detoxification system is a vital mechanism against toxic compounds, oxidative agents and free radicals, including ROS. This system consists of non-enzymatic and enzymatic mechanisms. The non-enzymatic mechanism utilizes the antioxidant substrates that bind to toxic compounds or interrupt free radical chain reactions, and finally reduce the harmful or reactivity. Examples of non-enzymatic antioxidants are vitamin C, vitamin E, carotenoids, polyphenols and cyanobacterial phycobiliproteins (Nimse & Pal, 2015). Enzymatic reaction is another route to

scavenge ROS in the cell using detoxification and antioxidant enzymes. These enzymes catalyze the conjugation, oxidation-reduction, transport-excretion or other mechanisms to reduce reactivity of substrates. Finally, the toxic/reactive compounds are converted into stable form and eliminated out of cells (Mol et al., 2017). Examples of detoxification and antioxidant enzymes are catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPX) and glutathione s-transferase (GST).

GST superfamily is a set of enzymes involved in cellular detoxification process. They catalyze conjugation between glutathione and either xenobiotic substrates or ROS. The conjugated product from this reaction is less reactivity and less harmful. Finally, this product is further eliminated or neutralized, associated with other detoxification enzymes (Singh et al., 2018). Thus, GST is one of the essential and well-acceptable detoxification enzymes responding against stress factors. Moreover, GST is also widely applied for biotechnological approaches; for instance, bioremediation, agricultural biotechnology, medical application and nanotechnology (Perperopoulou et al., 2018).

*Halothece* sp. PCC7418 is a halophilic and halotolerant cyanobacterium which was originally isolated from the Dead sea, Israel. This extremophile can thrive under high salinity up to 3.0 M NaCl at alkali pH up to 11 (Kageyama et al., 2011; Waditee-Sirisattha et al., 2014). Therefore, the cellular detoxification enzymes would be involved in cellular homeostasis and responses under stress condition. Based on public database, Kyoto Encyclopedia of Genes and Genomes (KEGG) (www.genome.jp/kegg/), there are four putative GSTs in this cyanobacterium. The patterns of transcript accumulations for these *Halothece* GSTs were previously investigated. Three *Halothece* GSTs were up-regulated under salt- and oxidative stresses (Kortheerakul, 2019). In this thesis, functional analysis and characterization were performed. The results obtained in this study would contribute to the understanding of cellular detoxification and adaptation under stress condition in extremophilic cyanobacteria. Furthermore, these results might be applied in biotechnology approaches in the future.

**The objective of this research**

1. To examine the physiological role of GST under salt stress and oxidative stress conditions using recombinant *Escherichia coli* cells
2. To functionally characterize *Halothece* GST

**Hypotheses of this research**

1. GST is one of crucial enzymes for cellular detoxification in the extremophilic cyanobacterium *Halothece* sp. PCC7418.
2. Besides the glutathione transferase activity, *Halothece* GST might consist of peroxidase activity against $H_2O_2$ as additional features.

# CHAPTER II
# LITERATURE REVIEWS

## 2.1 Extremophilic cyanobacteria

Cyanobacteria, an enormously diverse group of prokaryotes, are oxygenic phototrophs that ubiquitously inhabit our planet. They are among the first microorganisms to inhabit Earth according to fossils dating back 3.5 billion years (Demoulin et al., 2019). As same as other bacteria, their cell composed of peptidoglycan cell walls, 70S ribosomes and circular DNA as genomic material (Nguyen & Hoang, 2016). Although cyanobacteria are photosynthetic organisms but no chloroplasts. Cyanobacterial photosynthetic pigments include chlorophyll a, chlorophyll b, carotenoid, and other chromoproteins known as phycocyanin, allophycocyanin and phycoerythrin. Chromoproteins are organized in the phycobilisomes, located in cytoplasm (Elanskaya et al., 2018).

Among microbial world, cyanobacteria are unique because they grow in diverse habitats. According to photosynthetic ability and a variety of biosynthesis pathways, cyanobacteria play many beneficial roles in environments. For instance, they are producers in aquatic and marine ecosystems, and also symbiosis with other aquatic or marine organisms, such as diatom and sponge (Andreeva et al., 2020). Moreover, they are also utilized as human foods and source of precursors for industrial biotechnology. For instance, the cyanobacterial-based production of valuable sugar (Frigaard, 2018) and a source of mycosporine-like amino acids (MAAs), used as antioxidant and UV-screening compound (Tarasuntisuk et al., 2019). It should be noted that some cyanobacteria can cause environmental issues, such as algae bloom. Some of them produce toxic substrates known as cyanotoxins (Puschner & Moore, 2013). As aforementioned that cyanobacteria are unique among the microbial world because they grow in diverse habitats, which in many cases are extreme. Extreme environments are widespread on Earth, encompassing vary distinct regions, including hypersaline lakes, hot springs, deserts, volcanoes, and polar regions (Bolhuis et al., 2014; Hagemann, 2011; Thomas et al., 2005). Extremophiles are organisms that have the ability to endure at least one extreme environmental condition. They are

primarily prokaryotes with few eukaryotic members. In this thesis, we focus on the extremophilic cyanobacteria.

The extremophiles not only tolerate to the extreme condition but also require some substrates or conditions found in that extreme environment to grow. They can be broadly divided into subtypes according to their extreme habitats. There are thermophiles, psychrophiles, halophiles, barophiles and acidophiles/alkaliphiles. A list of representative extremophilic cyanobacteria and their habitats is summarized in Table1. Thermophiles refer to extremophilic cyanobacteria inhabit in high temperature ecological niches, such as hot spring and marine volcano (Amarouche-Yala et al., 2014). Psychrophiles are capable of growing under cold temperatures, with optimal growth temperatures ranging from 5-20°C, such as south pole (Nadeau & Castenholz, 2000). For halophiles, these are microbes inhabit in high salinity environments, such as salt or alkali lake (Yang et al., 2020). In case of barophiles, these are microbes withstand under high pressure environments (Rampelotto, 2013). Lastly, acidophiles/alkaliphiles are capable of growing in low or high pH area (Berry et al., 2003; Steinberg et al., 1998).

Extremophiles are facing to the fluctuated stress factors in their extreme habitats. Thus, their metabolic pathways or cellular detoxification systems have been developed and/or evolved for maintenance of cellular homeostasis, ionic balance and oxidative status, that make them can survive. Besides, extremophiles are known as important sources of distinct enzymes, the so-called extremozymes. They can be functioned under non-optimal condition, suitable to use in industrial processes and able to apply in other research approaches.

**Table 1** A list of representative extremophilic cyanobacteria and their habitats

| Cyanobacteria | Extremophilic categories | Habitats | Reference |
|---|---|---|---|
| *Halothece* sp. PCC7418 | Halophile | Dead sea, Israel (~ 3.0 M NaCl) | (Waditee et al., 2005) |
| *Euhalothece* sp. Z-M001 | Halophile | Salt Lake in Africa (>7% NaCl) | (Yang et al., 2020) |
| *Dactylococcopsis salina* | Halophile | Salt Lake in Sinai, Egypt (7-18% NaCl) | (Walsby et al., 1983) |
| *Coleofasciculus* sp. | Halophile | Salt Lake in Sinai, Egypt (7-18% NaCl) | (Oren, 2015) |
| *Pleurocapsa* sp. | Halophile | Salt Lake in Sinai, Egypt (7-18% NaCl) | (Oren, 2015) |
| *Leptolyngbya hypolimnetica* | Halophile, Alkaliphile | Hot lake, Washington, USA ($MgSO_4$ >10%, pH ~ 8.5) | (Lindemann et al., 2013) |
| *Nodularia* sp. | Halophile | Great Salt Lake, Utah USA, (6-10% NaCl) | (Roney et al., 2009) |
| *Gloeomargarita* sp. | Thermophile | Hot spring in Algeria (> 50°C) | (Amarouche-Yala et al., 2014) |
| *Thermosynechococcus elongatus* BP-1 | Thermophile | Hot spring in Japan (~ 55°C) | (Nakamura et al., 2002) |
| *Synechococcus* sp. | Thermophile | Hunter's Hot Spring, Oregon, USA (~ 85°C) | (Miller & Castenholz, 2000) |
| *Stanieria* sp. HS-29 | Thermophile | Hot spring in Indonesia (30-50°C) | (Prihantini et al., 2016) |
| *Cyanothece* sp. HKAR-1 | Thermophile | Hot spring in India (~ 50°C) | (Rastogi et al., 2012) |
| *Nostoc* sp. HKAR-2 | Thermophile | Hot spring in India (~ 50°C) | (Rastogi et al., 2012) |
| *Scytonema* sp. HKAR-3 | Thermophile | Hot spring in India (~ 50°C) | (Rastogi et al., 2012) |
| *Rivularia* sp. HKAR-4 | Thermophile | Hot spring in India (~ 50°C) | (Rastogi et al., 2012) |
| *Gloeocapsa* sp. PCC7428 | Thermophile | Hot spring in Sri Lanka (50-60°C) | (Mukaiyama et al., 2019) |
| *Oscillatoria* spp. | Psychrophile | Antarctic meltwater ponds (< 8°C) | (Nadeau & Castenholz, 2000) |
| *Limnothrix* sp. | Acidophile | Acidic lake in mining district, Germany (pH < 4.5) | (Steinberg et al., 1998) |
| *Arthrospira platensis* | Alkaliphile | Alkali lake in East Africa (pH 9-12) | (Berry et al., 2003) |

### 2.2 Stress environment of the Dead Sea

Extreme environments should be considered as habitats characterized by harsh environmental conditions, beyond the optimal range for the normal organism to live; however, the extremophilic organisms, which were mentioned in section 2.1, can thrive. Extreme environments encompass vastly diverse terrains, such as hypersaline lakes, hot springs, deserts, volcanoes, and polar regions (Bolhuis et al., 2014; Gómez, 2011; Hagemann, 2011; Thomas et al., 2005). These environments are also the stress factors affecting the microbial cells. In some environments contain more than one stress factors at a time. For example, salt lake contains more than 5-10% NaCl, thus it is regarded as salt-stress environment. At this salt concentration, it also causes the osmotic stress to the cells. Taken together, these are ionic and osmotic stresses. Each stress factor has different pattern to affect the cells, but sometimes it consequentially leads to secondary stress, consequentially.

The Dead Sea is one of the most well-known salt lake located between Israel and Jordan. It is also a lowest elevation on land in the world, more than 420 meters below sea level (Avriel et al., 2011). This lake contains extremely high salt concentration, approximately 34% (W/W) which is higher than ocean salinity about 10 times. Moreover, UV radiation in this area is also high (Jacob et al., 2017). Therefore, the Dead Sea is regarded as one of the most extreme environments in the world. A list of stress conditions existing in the Dead Sea area and their impacts are described below.



#### 2.2.1 Salt stress

Salt stress is regarded as one of the abiotic stress factors mostly found in the world. High salt concentrations interfere cellular ionic balance and causes the accumulation of misfolded and unfolded proteins *in vivo*. The consequent effects of ionic disruption cause ROS production, membrane disruption as well as electron transport disruption. In cyanobacteria and microalgae, the carotenoid content, which is an antioxidant against ROS, was found to be increased under salt/osmotic stress treatment. This evidence is one of examples to suggest that high salt concentration is related to ROS production, and elevated ROS level finally resulted in oxidative stress

(Pancha et al., 2015). Salt stress coincident with osmotic stress and ion toxicity sometimes can lead to cell damages (Wang et al., 2011). On the other hands, the stress factors are the signal regulating gene expression and cellular metabolisms. Salt stress triggers the morphological changes and physiological adaptation, such as lipid content accumulation and decreasing of carbon metabolism (Wang et al., 2016). These adaptations make cells are more compatible to survive under stress conditions (Gandhi & Shah, 2016).

### 2.2.2 Osmotic stress

The environment containing high salt concentration may cause the extreme ionic strength. Thus, it is hypertonic condition surrounding the cells that causes the water osmosis out of cells. The osmotic pressure also causes cell shrinkage and ionic disruption. In addition, a sudden osmotic upshift affects a water efflux from the cells, loss of turgor pressure, and reduced cell growth (Brautaset & Ellingsen, 2011).

### 2.2.3 Stress caused by ultraviolet

Ultraviolet (UV) is a radiation covers the wavelength range of 200–400 nm, which is high frequency and energy than visible wavelength. UV can be divided into 3 ranges, UV-A (315-400 nm), UV-B (280-315) and UV-C (200-280 nm), respectively. Amongst these ranges, UV-C dissipates the highest energy. Fortunately, UV-C range is completely absorbed by the ozone and other gases in atmospheric layer. UV-B and UV-A are partially absorbed too. Thus, UV-B dissipates the highest energy and also the most harmful range that can hits the Earth's surface (Blaustein & Searle, 2013). UV-B can damage cells directly and can cause skin cancer in human, while UV-A is less harmful but also cause mutation and indirect damage to DNA. In cyanobacteria, mild dose exposure of UV radiation promotes the photosynthetic ability; however, high dose exposure of UV turned to be harmful. UV-B affects DNA and protein structures, pigment accumulations, activity of metabolic pathway and cellular morphology (Rastogi et al., 2014). The defenses mechanism against UV of cyanobacteria were developed in many ways. For example, production of sunscreen compound, such as mycosporine-like amino acids (MAAs) and scytonemins. These

compounds screen and protect cyanobacterial cells from excess UV exposure (Pathak et al., 2019; Tarasuntisuk et al., 2019). Unfortunately, UV radiation not only affects the cell components directly as mentioned above, but UV also excites the photosystem and causes the oxidative stress. The saturation of photochemistry leads to the accumulation of excitation energy in the pigment bed, finally resulted in the generation of ROS by energy transfer (Pathak et al., 2019). The accumulation of ROS caused by UV also affects cellular components and oxidative balance, as well as the consequence of salt stress.

### 2.2.4 Oxidative stress

ROS are groups of highly reactive molecules which are both taken up directly from environment and spontaneously generated in cell. There are many forms of ROS, such as superoxide anion ($O_2^{-\cdot}$), singlet oxygen ($^1O_2$), hydroxyl radical (•OH), hydrogen peroxide ($H_2O_2$), hypochlorous acid (HOCl), peroxyl radicals (ROO•) and hydroperoxyl radicals (HOO•). Of these, the $O_2^{-\cdot}$, $H_2O_2$ and •OH have the most significant effects to cellular physiological processes (Birben et al., 2012). The *in vivo* ROS generation is spontaneous process and occurs from aerobic cellular metabolism. In normal case, $^1O_2$ can be generated from adding energy to oxygen from photosensitized chlorophyll. But in case that light intensity is higher than normal, which cannot be handled by the capacity of photosynthetic electron transport chain, other ROS can be generated and resulted in an inactivation of the photosystems (Latifi et al., 2009). Moreover, since the cells are triggered by other stress factors, such as high dose of UV radiation and high salt concentration, ROS can be excessively generated in the cells (Birben et al., 2012; Pathak et al., 2019). The other oxidant forms that significantly affect the cell are Reactive Nitrogen Species (RNS). Similar to ROS, the RNS (such as nitric oxide (NO) and its derivatives) are highly reactive molecules that can react defectively to the cell components (Alhasawi et al., 2019). Both ROS and RNS can disrupt cellular metabolism, as known as oxidative stress and nitrosative stress, respectively (Kurutas, 2016).

Oxidative stress is termed as the imbalance condition between oxidants (free radicals and ROS) and antioxidants. When ROS and free radicals are accumulated higher than antioxidants, the excess can cause the defective cellular reactions and resulted in either cellular components damages or alteration of their functions. There are many reports suggesting that ROS are the main molecules affect the cellular components. $H_2O_2$ and $^1O_2$ inhibit the repair process of photosystem II in the cyanobacterium *Synechocystis* sp. PCC 6803 by suppressing the translation of elongation of protein D1 (Nishiyama et al., 2004). The iron-sulfur clusters can be oxidized by $O_2^-$, resulting in inactivation of related enzyme. (Imlay, 2003). In addition, ROS and free radicals can attack the polyunsaturated fatty acids (PUFA), on cell membrane, by lipid peroxidation. This reaction generates fatty acid radical and then immediately adds oxygen to form a fatty acid peroxyl radical. Thereafter, the continuous chain reaction caused by fatty acid peroxyl radicals react other lipid molecules and break down cellular components (Nimse & Pal, 2015). ROS also affect to structural proteins by either cross-linking or fragmentation on polypeptide chains as well as alteration of electrostatic charges, and oxidizing of amino acids. Moreover, oxidative stress affects the signal transduction in some organisms by defective reaction with signal proteins or receptors (Birben et al., 2012).

Oxidative stress is one of the most significant stress factors causing cell damages and cell death. Moreover, other stress conditions, such as salt stress and stress from UV radiation, are also consequentially triggered the generation of ROS. These free radicals may affect the cellular homeostasis and metabolism, by attacking the cellular structures, inhibiting metabolic pathways or causing defective reactions. Thus, the defense mechanism against ROS/RNS and other free radicals is a vital process to maintain oxidative balance between cellular oxidants and antioxidants, and prevent the defective reactions caused by ROS that finally resulted in cell death.



**2.3 Cellular detoxification system**

This is a vital process to maintain cellular homeostasis and oxidative balance, as well as defense against toxic substrates, xenobiotics, and the excess free radicals. This process also includes ROS generation that is triggered by stress factors. As we

known that free radicals are harmful and causes many defective reactions in cells. On the other hand, free radicals in appropriate level of free radicals are useful for cell signaling and induction/regulation a number of cellular metabolic pathways (Kurutas, 2016; Poljsak et al., 2013). Thus, the free radicals and ROS level must be controlled in appropriate level. The key mechanism for this scenario is balance of free radicals and antioxidants, using cellular detoxification. This is the complex system, even in prokaryotes. Overall processes can be divided into two main mechanisms. There are non-enzymatic and enzymatic antioxidant systems (Mol et al., 2017).

### 2.3.1 Non-enzymatic system

This system associated with a number of non-enzymatic antioxidants. They act as the substrates that bind to toxic compounds or interrupt free radical chain reactions and reduce the harmful or reactivity directly (Nimse & Pal, 2015). Representative compounds and brief mechanisms are given below.

#### 2.3.1.1 Vitamin E

Vitamin E ($\alpha$-tocopherol) is one of well-known antioxidants, regarding as a shield against oxidative stress. It is a soluble lipid that can bind to lipid peroxyl radicals ($LOO^{\cdot}$) generated during lipid peroxidation. The product tocopheroxyl radical is more stable and cannot react with other lipids. Therefore, the chain reaction of lipid peroxidation is stopped by this “chain breakers” (Nimse & Pal, 2015).

#### 2.3.1.2 Vitamin C

Vitamin C (ascorbic acid) is a water-soluble compound. It has been recognized as one of the most well-known antioxidants. To repair the defected lipids and terminate lipid peroxidation, vitamin C changes to the ascorbate radical and donates an electron to lipid radicals, turning them to the stable forms. Thereafter, two ascorbate radicals immediately react themselves, resulting in one molecule of ascorbic acid and one molecule of dehydroascorbate. Finally, the dehydroascorbate is converted back to the ascorbic acid by adding two electrons, using enzyme

oxidoreductase (Nimse & Pal, 2015). Moreover, vitamin C and vitamin E work together as a partner in defense mechanism. Membrane-bound vitamin E can be oxidized and inactivated. Then, vitamin C causes significantly regeneration and repair of oxidized vitamin E by non-enzymatic mechanism (Chan, 1993).

#### 2.3.1.3 Vitamin B12

Vitamin B12 (cobalamin) is also a water-soluble compound regarding as one of antioxidants against oxidative stress. Vitamin B12 possesses the ability to scavenge ROS superoxide in particular. In addition, vitamin B12 indirectly defenses against ROS by promoting the accumulation of glutathione, another antioxidant related to enzymatic mechanisms, to reduce the reactivity of ROS via glutathione-conjugation. In human, vitamin B12 modulates the production of cytokines and growth factors offering the protection from immune responses, induced by oxidative stress (van de Lagemaat et al., 2019).

#### 2.3.1.4 Flavonoids

These are a group of the natural benzo-γ-pyran derivatives, which can be found in various organisms, mainly in plants. Flavonoids can be broadly divided into seven groups based on their ring structures, including flavones, flavonols, flavanones, flavanonols, flavanols or catechins, anthocyanins and chalcones (Panche et al., 2016). Substantial evidence suggests that flavonoids exert strong antioxidant activities. Various kinds of flavonoids, such as rutin, catechin, and naringin, are able to scavenge ROS and protect DNA from cleaving or damaging induced by the hydroxyl radicals (Russo et al., 2000). Anthocynidine, another class of flavonoids, can donate electron to free radical and scavenge ROS. Moreover, anthocyanidine associated with metal ion-chelating activity can inhibit lipid peroxidation (Pekkarinen et al., 1999). Forming complex between flavonoids and metal, such as copper and iron, also resulted in prevention of the excess ROS generation in cells (Nimse & Pal, 2015).

#### 2.3.1.5 Carotenoids

Carotenoids are versatile C-40 isoprenoid compounds synthesized by plants, algae, and bacteria. The members include β-carotene, lycopene, zeaxanthin, astaxanthin and lutein (Young & Lowe, 2018). Carotenoids has ability to scavenge the ROS, especially peroxyl radicals. The peroxyl radicals are generated during lipid oxidation. Thus, carotenoids play a vital role to prevent the damages of lipids and lipoproteins on the cell wall (Stahl & Sies, 2003). Astaxanthin activity is regarded as one of the most powerful antioxidants known to date, which was widely used in medical approaches and commercial cosmetic products. In addition, astaxanthin also possesses anti-inflammatory, anti-aging and antiproliferative ability (Sztretye et al., 2019). Lycopene and β-carotene are the effective carotenoids for antioxidants. Lycopene exhibits the strongest ability to quench singlet oxygen, followed by the β-carotene (Nimse & Pal, 2015; Rao & Rao, 2007).

#### 2.3.1.6 Phycobiliproteins

Phycobiliproteins are the groups of water-soluble chromophore protein complex derived from cyanobacteria and microalgae. According to the unique photosynthetic ability of cyanobacteria and microalgae, phycobiliproteins are utilized for light absorption in another wavelength, apart from chlorophyll a. These complex proteins consist of phycoerythrin, phycocyanin and allophycocyanin (absorption maxima lie between 490–570 nm, 610-625 nm, and 560-660 nm, respectively) (Pagels et al., 2019; Rajalakshmi, 2018). Moreover, phycobiliproteins also exert antioxidant ability against free radicals. There are a number of evidence suggest that phycobiliproteins scavenge various kinds of ROS and inhibit excessive generation of ROS in cells (Kim et al., 2018; Riss et al., 2007; Sonani et al., 2015).



### 2.3.2 Enzymatic systems

The cellular detoxification system using enzymes is one of the most important mechanisms in cells, to maintain cellular redox homeostasis and survive from toxic substrates derived from the stress environments. These enzymes performed specific catalytic activity to specific target. However, all enzymatic

reactions are related and linked together as a metabolic pathway, resulted in systematic detoxification and elimination of toxic substrates, step-by-step. There are various kinds of toxic substrates and stress factors affect to the cell, not only free radicals and ROS, but also metal ions and xenobiotic substrates (Burgos-Aceves et al., 2018). In addition, ROS affects the cellular components and turn some biomolecules into radicals, such as lipid radicals generated from lipid oxidation, caused by the ROS (Nimse & Pal, 2015). Therefore, a number of enzymatic detoxifications and antioxidant mechanisms have evolved to deal with each toxic substrate, and each situation, specifically and systematically. Enzymatic detoxification systems can be classified into three main groups. These are antioxidant enzymes, phase I detoxification enzymes and phase II detoxification enzymes (Rougée et al., 2014; Yang et al., 2011).

#### 2.3.2.1 Antioxidant enzymes

Antioxidant enzymes are involved in the elimination or neutralization of ROS by catalyzing reaction to scavenge or inhibit directly. These enzymes function in different subcellular compartments. Although there are various antioxidant enzymes in living organisms, but at least six essential antioxidant enzymes that are ubiquitously found, which are catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase, ascorbate peroxidase and dehydroascorbate reductase (Das & Roychoudhury, 2014; Singh et al., 2018; Yang et al., 2011). Brief catalytic activity of each enzyme is demonstrated in Table 2.



##### 2.3.2.1.1 Catalase (CAT)

CAT (E.C. 1.11.1.6) is the first antioxidant enzyme discovered since 1990s. This enzyme presents in all aerobic organisms. CAT structure is tetrameric protein containing either $Fe^{2+}$ or $Fe^{3+}$ as a core metal (Wu et al., 2014). It is particularly localized in cytosol and other $H_2O_2$ production-related organelles, in higher eukaryotes. CAT efficiently catalyzes $H_2O_2$ into $O_2$ and $H_2O$, with very high turnover rate ($6 \times 10^6$ molecules of $H_2O_2$ to $H_2O$ and $O_2$ per minute). However, it is

less specificity to other organic peroxides (Das & Roychoudhury, 2014; Sharma & Ahmad, 2014).

**2.3.2.1.2 Superoxide dismutase (SOD)**

SOD (E.C. 1.15.1.1) is the metalloenzyme, ubiquitously presented in all aerobic organism. SOD can be classified into three groups (Fe-SOD, Mn-SOD and Cu/Zn-SOD) based on its metal-core. This enzyme has regarded as the first line of defense against defective reaction caused by ROS. SOD mainly catalyzes the dismutation of $O^{\bullet-}_2$ and turns into $O_2$ and $H_2O_2$ (Das & Roychoudhury, 2014). $H_2O_2$ from the first reaction is further eliminated by other enzymes, such as CAT (described in section 2.3.2.2.1). SOD has also reported in responses to abiotic stress in plants (Szőllősi, 2014).

**2.3.2.1.3 Glutathione peroxidase (GPX)**

GPX (E.C. 1.11.1.9) is the antioxidant enzyme particularly localized in cytoplasm and mitochondria. This enzyme plays a role in glutathione-associated reaction to scavenge or turn some ROS into a more stable form (Mulgund et al., 2015). GPX catalyzes reaction between reduced glutathione (GSH) and lipid peroxide to produce stable lipid, $H_2O$ and oxidized glutathione (GSSG). Moreover, GPX also catalyzes the reaction between GSH and $H_2O_2$ to generate $H_2O$ and GSSG (Higuchi, 2014).



**2.3.2.1.4 Glutathione reductase (GR)**

GR (E.C. 1.6.4.2) is not directly involved in ROS scavenging, but it plays a role as glutathione recover machinery. As described in section 2.3.2.1.3, the ROS scavenging reaction using glutathione-associated reaction also produces GSSG in the end. To maintain the cellular balance of GSH/GSSG, GR is in responsible to reduce one molecule of GSSG back to two molecules of GSH, using one NADPH. These GSHs are also available for reuse in ROS scavenging reaction again (Das & Roychoudhury, 2014).

#### 2.3.2.1.5 Ascorbate peroxidase (APX)

APX (E.C. 1.1.11.1) is an antioxidant enzyme with the similar function as CAT but it catalyzes the reaction similar to GPX. APX catalyzes the oxidize reaction to scavenge $H_2O_2$ and turn into $H_2O$ and dehydroascorbate (DHA). This reaction uses ascorbic acid (AA) as reducing agent. However, this enzyme particularly found in higher eukaryotes, especially in plants. In prokaryotes may be found in cyanobacteria (Pandey et al., 2017a; Pathak et al., 2019).

#### 2.3.2.1.6 Dehydroascorbate reductase (DHAR)

DHAR (E.C. 1.8.5.1) is an antioxidant enzyme with consequent function after APX. Similar to GSSG recovering by GR, the DHA in which produced from the reaction catalyzing by APX can be recovered back to ascorbic acid (AA), catalyzed by DHAR. AA product is available for reuse in $H_2O_2$ scavenging reaction again. This reaction uses two molecules of GSH and also generates one molecule of GSSG (Das & Roychoudhury, 2014). The chemical equation is shown below.

**Table 2** Catalytic activity of the representative antioxidant enzymes

| Enzyme | Catalytic activity |
|---|---|
| CAT | $2H_2O_2 \longrightarrow 2H_2O + O_2$ |
| SOD | $O^{\bullet-}_2 + O^{\bullet-}_2 + 2H^+ \longrightarrow 2H_2O_2 + O_2$ |
| GPX | $H_2O_2 + GSH \longrightarrow H_2O + GSSG$ |
| GR | $GSSG + NADPH \longrightarrow 2GSH + NADP^+$ |
| APX | $H_2O_2 + AA \longrightarrow 2H_2O + DHA$ |
| DHAR | $DHA + 2GSH \longrightarrow AA + GSSG$ |



#### 2.3.2.2 Phase I detoxification enzymes

Detoxification enzymes are the other parts of cellular detoxification system. These enzymes mainly involved in the cellular detoxification of toxic substrates and xenobiotics that can cause the generation of ROS and lead to oxidative stress later. Phase I detoxification associated with the transformation of

toxic substrates to less harmful forms and able to be detoxified or eliminated by phase II detoxification reaction subsequently (Yang et al., 2011). In case of phase I detoxification is malfunction, the toxic substrates are not suitable for the further detoxification in phase II. There are many enzymes in this group, such as alcohol dehydrogenase, aldehyde dehydrogenase, cytochrome P450s and aldo-keto reductases (Mol et al., 2017).

**2.3.2.2.1 Cytochrome P450s (CYP)**

CYPs (E.C. 1.14.1.1) exerts monooxygenase activity. They participate in the oxidation and metabolism of various xenobiotics and endogenous toxic substrates by converting them into $H_2O$ and $O_2$. This group is regarded as one of the key enzymes for phase I detoxification (Wang et al., 2006). CYPs are mostly found in almost living organisms with a high diversification. To date, CYPs can be classified into at least 18 families and 44 sub-families, based on their sequence homology and putative protein function (Shankar & Mehendale, 2014). In cyanobacteria, CYPs perform potentially for biomolecule biosynthesis. For instance, CYPs from *Nostoc* spp. together with other enzymes participate in bioproduction of germacrenes, a group of volatile organic hydrocarbon with antimicrobial and insecticidal properties (Robert et al., 2010).



**2.3.2.2.2 Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH)**

ADH (E.C. 1.1.1.1) and ALDH (E.C. 1.2.1.2) belong to the group of enzymes widely distribute in both prokaryotes and eukaryotes. These enzymes particularly catalyze the oxidation/reduction of various alcohols and aldehydes. For detoxification ability, ADH catalyzes oxidation of the alcohol, which can be toxic to cells, to be secondary toxic substrates, such as acetaldehyde. After that, this compound is further oxidized to non-toxic acetic acid (Lu et al., 2020).

**2.3.2.2.3 Aldo-keto reductases (AKR)**

AKRs involved in the reduction of aldehydes and ketones to the primary and secondary alcohols, respectively. This mechanism is useful for detoxification of carbonyl toxic compounds, such as melandialdehyde (MDA) and methylglyoxal (MG) (Vemanna et al., 2017). This group of enzymes also widely presented in almost all organisms. To date, AKRs can be classified into at least 16 families based on sequence diversity (Ellis, 2002; Penning, 2015).

**2.3.2.3 Phase II detoxification enzymes**

Phase II detoxification is a subsequent mechanism after phase I detoxification. In this phase, the less toxic and more water-soluble substrates whose derived from phase I metabolism are further elimination and/or degradation. Moreover, some kinds of ROS that are taken up from the environment or generated during the cellular metabolisms, such as $H_2O_2$ and lipid radical, are also in responsible of phase II detoxification associated with antioxidant enzymes and non-enzymatic antioxidants (Hossain et al., 2015). There are various reactions involved in phase II detoxification, including conjugation, acetylation, methylation, glucuronidation and sulfation. There are also many enzymes involved in phase II detoxification, for example glutathione s-transferase, N-acetyl-transferase and methyl-transferase (Yang et al., 2020).



**2.3.2.3.1 Glutathione s-transferases (GSTs)**

These are a set of enzymes involved in detoxification of xenobiotic substrates and ROS. They are ubiquitously presented in all living organisms. According to the diversity of GST, these group of enzymes can be divided into four superfamilies, based on their subcellular localization. There are cytosolic GST, mitochondrial GST, microsomic membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) and fosfomycin resistance protein (Theoharaki et al., 2019). Typically, GSTs catalyze the conjugation between glutathione and electrophilic substrates, forming the conjugated products. These products are less reactivity and less toxicity, then are eliminated or degraded by other subsequent

mechanisms. Thus, glutathione-conjugation is one of the key reactions in phase II detoxification (Yang et al., 2020; Zhang et al., 2018).

**2.3.2.3.2 Sulfotransferases (SULTs)**

SULTs (E.C. 2.8.2.16) are a group of important enzymes in cytosol. They are highly diverse and presented in all organisms. SULT catalyzes the formation of sulfuric acid esters, mostly referred to sulfates, from a wide range of xenobiotics and their endogenous toxic metabolites. SULT has shown to play a role in cellular detoxification concerted with other phase II detoxification enzymes, such as MT. Some mono-conjugated products, usually methylated metabolites, are consequently sulfonated by SULT in their metabolism (Gamage et al., 2005; Suiko et al., 2017).

**2.3.2.3.3 Methyl-transferase (MT)**

MT (E.C. 2.1.1.57) is the enzyme that transfers methyl groups to their substrates which can be metabolic precursors, xenobiotics, drugs and metallic substrates. The resulting reaction generates substrate methylation. These methylated products can be both precursors for other subsequent biosynthesis pathway and less toxic substrates for further detoxification steps, such as biomineralization or emission out of cells (Ranjard et al., 2003).

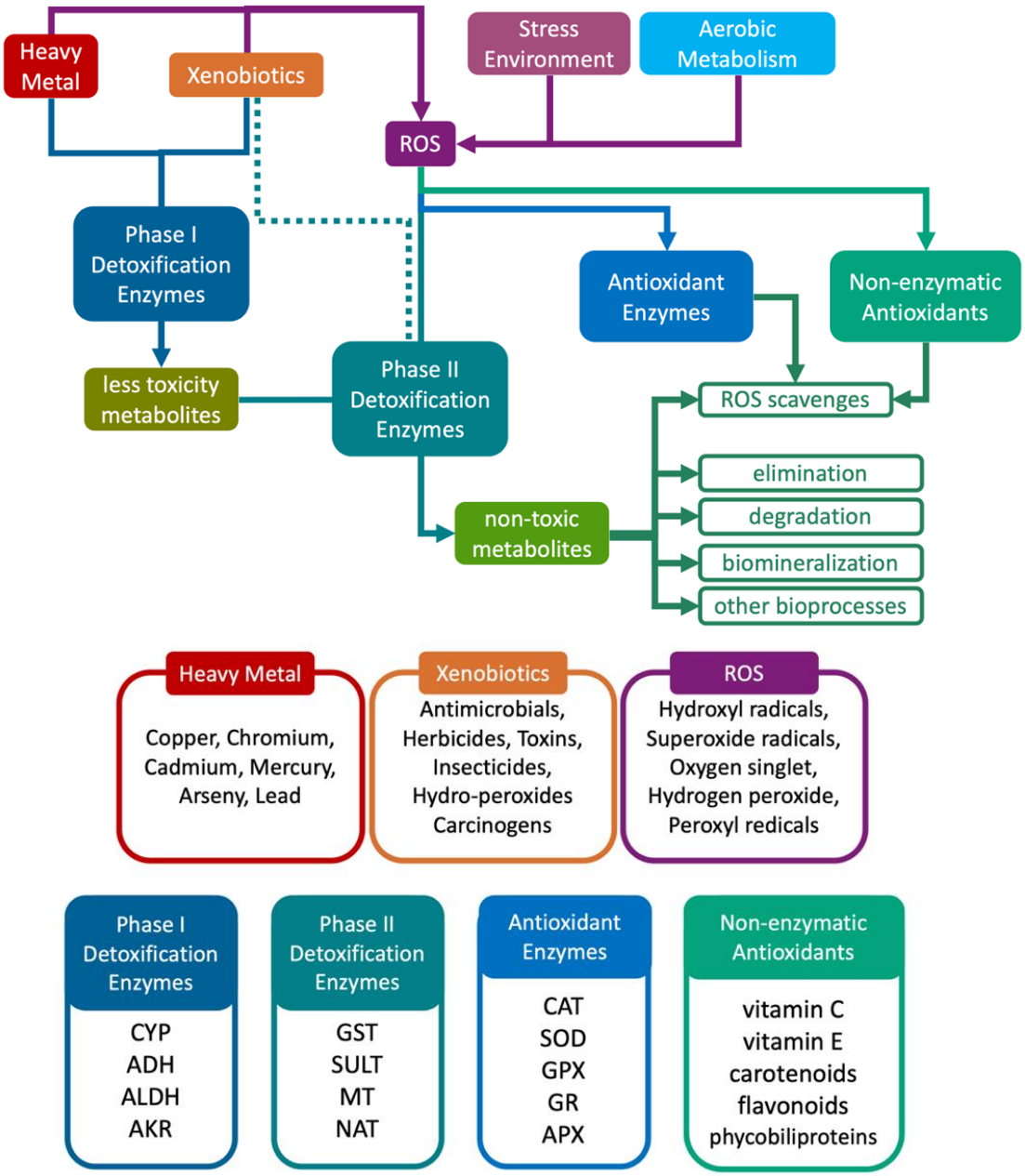


**Figure 1** A simplified diagram for the overview of cellular detoxification systems for each kind of toxic substrates. The example of the heavy metal, xenobiotic substrates, ROS, non-enzymatic antioxidants, antioxidant enzymes, phase I- and phase II detoxification enzymes are also shown (adapted from Rougée et al, 2014 and Yang et al, 2011).

## 2.4 Glutathione metabolism and glutathione s-transferase (GST)

### 2.4.1 Glutathione metabolism

Glutathione metabolism is another effective system used to scavenge and eliminate ROS. Based on KEGG database, it reveals that there are at least five main enzymes involved in this system. These include gamma-glutamyl-L-cysteine synthetase (GshA), glutathione synthetase (GshB), GPX, GR and GST. Glutathione is a tri-peptide molecule, i.e. glutamate, cysteine and glycine. Normally, glutathione is presented in reduced form (GSH) *in vivo*. The biosynthesis of GSH begins with the peptide bond forming between glutamate and cysteine by the function of GshA. Then, glycine is linked by the function of GshB (Pophaly et al., 2017). GSH can be used as antioxidant to directly scavenge ROS, using enzyme GPX. In some cases, GST also performs the ability to catalyze this reaction too (Roxas et al., 2000). This reaction oxidizes GSH and turn to be GSSG. To recover GSSG, GR is responsible to reduce GSSG back to the GSH. Another reaction with GSH is the conjugation, catalyzing by GST. The GSH can be conjugated to either xenobiotics, to reduce their toxicity and further detoxified by other related mechanisms, or cellular proteins, to protect them from defective reaction caused by ROS. In the second case, the glutathione conjugated proteins, as known as s-glutathionylated proteins, can be de-glutathionylated associated with GR or GPX (Mailloux et al., 2020; Zhang et al., 2018).

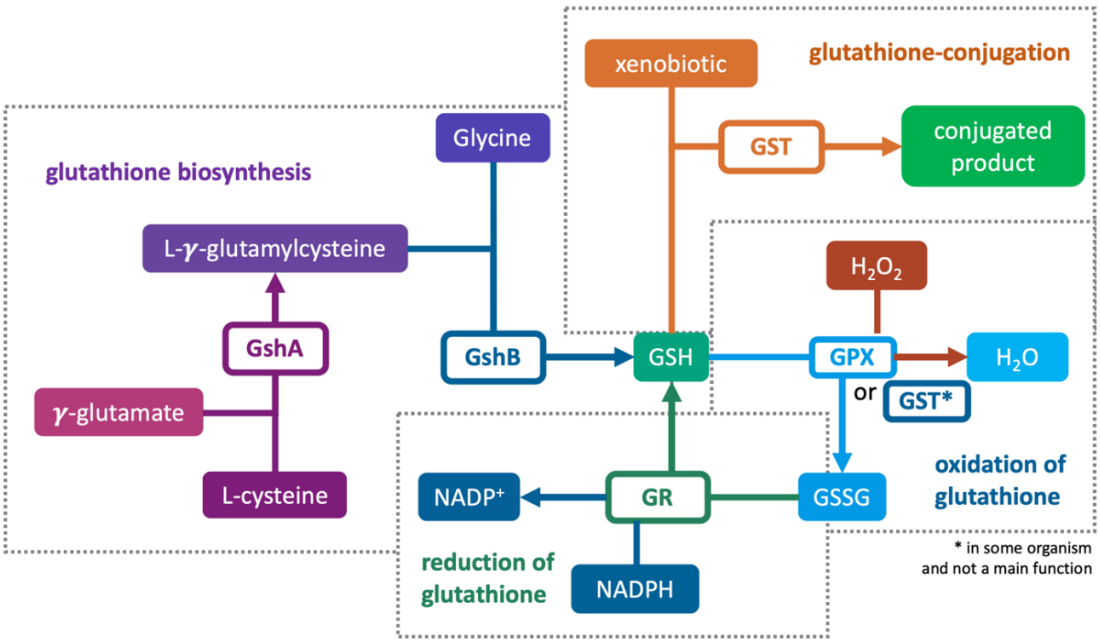


**Figure 2** A schematic diagram for glutathione metabolism and associated enzymes. Adapted from KEGG reference pathway (https://www.genome.jp/kegg-bin/show_pathway?map00480).

### 2.4.2 Glutathione s-transferase

Among antioxidant and detoxification enzymes, GST is one of the most important enzymes that involved in multicellular processes. They are ubiquitously present in all living organisms (Zhang et al., 2018). In bacteria, GST was firstly reported in *E. coli*. The *E.coli* GST was shown to be involved in structural modification of fosfomycin; the widely-used board spectrum antibiotic for both Gram-negative and Gram-positive pathogens (Falagas et al., 2016). GST plays the essential role in phase II detoxification of both xenobiotic substrates and the toxic metabolites. Metabolites are generated from the defective reactions, caused by ROS, such as DHA and lipid peroxidation end products (Nimse & Pal, 2015; Perperopoulou et al., 2018). Moreover, some previous researches reveal that GST in some organisms also directly scavenge ROS, such as $H_2O_2$, using peroxidase activity (Hossain et al., 2015; Pandey et al., 2017a; Roxas et al., 2000). GST is also crucial for cellular adaptation in several organisms against harsh environments. Thus, several GSTs, including bacterial GSTs, have been identified and extensively studied covering from

molecular structures, physiological roles, as well as applications in agricultural, medical, environmental and analytical biotechnologies (Perperopoulou et al., 2018).

#### 2.4.2.1 GST structure

All GSTs known to date are intracellular enzymes, having molecular mass approximately 26-30 kDa. The X-ray crystallography suggests that GST naturally forms as either homodimer or heterodimer (Shehu et al., 2019). Their structure typically consists of two domains. The first domain, locating at N terminus, contains glutathione binding site (G-site). Second domain is the electrophilic substrate binding site (H-site) which is located at C terminus. The G-site specifically binds to glutathione tripeptide molecule. Amino acid residues at G-site are highly conserved in all identified GSTs (Pophaly et al., 2017). In contrary, it has been shown that amino acid residues in the H-site are not conserved, but highly variable among species. Additionally, different GSTs can bind to various electrophilic xenobiotic substrates. It was evident that amino acid residues in H-site can cause special structure, such as a hair-pin and loop. These features lead to versatility of GST upon unique and/or harsh environmental conditions because the increased flexibility in GST structure can be functioned much better in special environmental conditions (Tossounian et al., 2019)

#### 2.4.2.2 GST classification



GST can be classified into four superfamilies based on their subcellular localization. These are cytosolic GSTs, mitochondrial GST, microsomic/membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) and fosfomycin resistance protein. Among these four superfamilies, mitochondrial GST is can be found only in eukaryotes, while fosfomycin resistance protein is presented only in some bacteria. Cytosolic GSTs are presented in all living organisms. In addition, cytosolic GSTs are the most diverse group and play various physiological roles in cells, such as stress tolerance, cellular apoptosis, secondary metabolite transportation and antibiotic resistance (Perperopoulou et al., 2018).

#### 2.4.2.2.1 Cytosolic GSTs

As mentioned above, cytosolic GSTs, which widely presented in all living organisms, are the most diverse group and play the essential roles in cellular detoxification and stress responses. To date, at least 18 subclasses of cytosolic GSTs were reported. These include Alpha-, Beta-, Delta-, Epsilon-, Zeta-, Eta-, Theta-, Iota-, Lambda-, Mu-, Nu-, Xi-, Pi-, Rho, Sigma-, Tau-, Phi- and Omega-class GST. The classification is based on several criteria, such as the conserved amino acid homology and phylogeny, substrate specificity, enzymatic activity and protein-protein interaction. Generally, the amino acid identity more than 40% is required to claim that these two GSTs in the same class (Pandey et al., 2017b; Theoharaki et al., 2019; Wiktelius & Stenberg, 2007). Some subclasses can be found in several organisms, such as Zeta-class and Theta-class GSTs. It should be noted that some subclasses are unique in certain organisms. For instance, Tau- class GST specifically presents in plants, whereas Beta-class GST was reported only in bacteria (Allocati et al., 2006). Some subclasses in some organisms contain unique characteristics, making special biochemical properties. For example, Beta-class GST consists of H-bond network in its structure that resulted in high catalytic efficiency to xenobiotic substrate and some antibiotics (Shehu et al., 2019). Unique features of Tau class GST were shown at the N-cap position in which the Ser/Thr residue was replaced by a glycine residue, resulted in suitable for some plant metabolisms (Allocati et al., 2006). Recently, Chi- and Rho-classes GST have identified in cyanobacterium *Synechocystis* sp. PCC6803 (Pandey et al., 2017b; Pandey et al., 2015b). Thus, discovery of novel GST isozyme is challengeable.

#### 2.4.2.2.2 Mitochondrial GST

Mitochondrial GST is the special GST group presented in eukaryotes. This group has the similar molecular weight with cytosolic GSTs. The amino acid sequences on the N terminus also share about 36% to the Theta-class GST. In contrary, mitochondrial GSTs still have unique protein folding, different from other cytosolic GSTs. According to these similarities and differences, sometimes mitochondrial GST can be called as Kappa-class GST, related to classification criteria

of cytosolic GSTs (Morel & Aninat, 2011). According to aerobic respiration, ROS are always spontaneously generated in mitochondria. Thus, mitochondrial GST are responsible for redox homeostasis, ROS scavenging and stress response, similar to cytosolic GSTs, but functions in eukaryotic mitochondria (Calabrese et al., 2017).

**2.4.2.2.3 MAPEG**

These GSTs function in eukaryotic organelle membranes and in bacterial microsomes. Alternatively, it can be called as microsomic GSTs. These can be classified into 4 subgroups, include subgroup I, II, III and IV. The amino acid similarity is less than 20% among subgroups. MAPEG has unique structure, different from both cytosolic and mitochondrial GSTs. (Bresell et al., 2005). The physiological roles of MAPEG are diverse, but particularly in cellular detoxification of toxic substrates, metabolism of eicosanoids and glutathione, and biosynthesis activity linked to other antioxidant mechanisms (Jakobsson et al., 2000).

**2.4.2.2.4 Fosfomycin resistant proteins**

Fosfomycin resistant proteins are the group of enzymes have been discovered since 1990s. Firstly, these proteins found to be involved in the resistance of fosfomycin in bacteria. Fosfomycin is a widely-used board spectrum antibiotic for both Gram-negative and Gram-positive pathogens (Falagas et al., 2016). This antibiotic inhibits bacterial MurA (UDP- NAG enolpyruvyl transferase), resulted in an inhibition of peptidoglycan synthesis and leads to cell wall disorder. However, many bacteria such as *E. coli, Klebsiella pneumoniae* and *Serratia marcescens*, possess the mechanism to overcome its mode of action by synthesis of resistant proteins. There are various forms of fosfomycin resistant proteins, which exhibit different mechanisms to inactivate the antibiotic. These can be classified into main four groups, include FosA, FosB, FosC and FosX (Huang et al., 2017). Among these four groups, FosA is found later that their mechanism against fosfomycin is similar to GST activity. FosA catalyze the conjugation of fosfomycin molecule and glutathione, resulted in structural modification. Finally, fosfomycin is inactivated (Bernat et al., 1997; Ito et al., 2017).

#### 2.4.2.3 GST catalytic activity

GST is one of the well acceptably enzymes for cellular detoxification by catalyzing the conjugation between glutathione and xenobiotic substrates to form the conjugated products. Consequently, these products become less reactivity, more soluble and more stable (Theoharaki et al., 2019). Nowadays, there are three main catalytic mechanisms of GST against the toxic substrates, based on what happen to glutathione during the reaction (Perperopoulou et al., 2018). The representative reaction for each catalytic mechanism was shown in Figure 3.

##### 2.4.2.3.1 Glutathione is consumed with product

This mechanism likely occurs in nucleophilic aromatic substitution reaction, nucleophilic substitution reaction and addition reactions of the xenobiotic modification. Glutathione molecule is consumed during the reaction, and never get free glutathione back at the end of reaction.

##### 2.4.2.3.2 Glutathione binds to substrate in intermediate level

This mechanism may be occurred in isomerization reactions and hydrolytic dehalogenations. Glutathione molecule is temporary consumed during the reaction, to form the intermediate. However, at the end of reaction, free glutathione is released from the product.



##### 2.4.2.3.3 Glutathione is oxidized

This mechanism may be occurred in the disulfide bond reduction, hydroperoxide reduction, thiocyanate reduction, reductive dehalogenation, dehydroascorbate reduction, and glutathionylation/ deglutathionylation cycle. In this mechanism, GSH (reduced glutathione) is oxidized. The electron is donated to toxic substrates, hydroperoxide substrates or ROS, to reduce their reactivity. In the end of reaction, GSSG (oxidized glutathione) can be reduced back to GSH using enzyme GR.

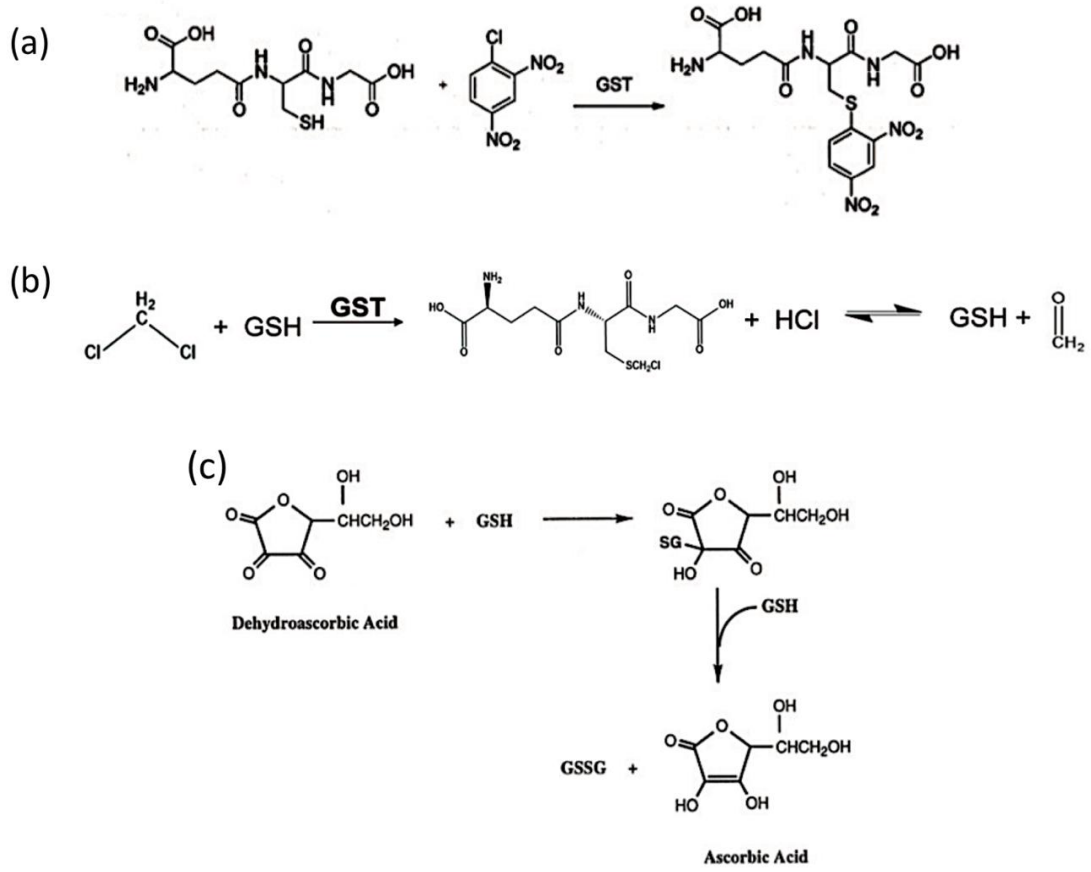


**Figure 3** The representative reaction catalyzed by GST: (a) mechanism that glutathione is consumed to form product, (b) mechanism that glutathione is temporary bound with substrate only in intermediate level, and (c) mechanism that glutathione is oxidized (Perperopoulou et al., 2018).



### 2.4.3 *Halothece* GSTs

*Halothece* sp. PCC7418 is the halophilic and halotolerant cyanobacterium isolated from the Dead Sea. This cyanobacterium can grow at concentrations up to 3.0 M NaCl and under alkaline pH up to 11 (Waditee-Sirisattha et al., 2014). High concentration of NaCl causes salt stress condition, induce the generation of ROS, and finally resulted in oxidative stress. Thus, this cyanobacterium possibly contains antioxidant and detoxification enzymes with special features, making the cell can survive and thrive under these extreme conditions. GST is one of the detoxification enzymes, having a major role in cellular homeostasis against oxidative stress. This

enzyme also possesses a potential for apply in various biotechnology approaches. Therefore, in the present study GST is selected for further study regarding the role in stress responses and its biochemical functions.

From bioinformatics analysis using KEGG database, there are at least four putative GST encoding genes in the entire genome sequencing of *Halothece* sp. PCC7418. These putative genes are PCC7418_0647, PCC7418_0729, PCC7418_1478 and PCC7418_3557. Hereafter, the GSTs encoded from these putative genes will be designated as GST_0647, GST_0729, GST_1478 and GST_3557, respectively. Bioinformatics analysis revealed distinct features of these putative GST encoding genes. The protein domain prediction using SMART program suggests that GST_3557, which comprised of a sole N domain, is significantly different from others. Amino acid sequences of three GSTs (GST_0647, GST_1478 and GST_3557 GST) were used to construct multiple alignment by ClustalW (the appendices). Homology is shown in the range of 12-24% similarity. The percentage of amino acid sequence similarity cutoff to determine the same class of GST was 30-40% (Pandey et al., 2017b). Thus, these three *Halothece* GSTs might be considered that they are the different group of cytosolic GSTs and/or having different properties.

Gene expression analysis of four putative GST encoding genes in *Halothece* sp. PCC7418 was previously performed under salt and oxidative stress conditions. Results revealed that there were differential expressions among these genes upon stresses (Kortheerakul, 2019). In this study, all four GST encoding genes from *Halothece* sp. PCC7418 were cloned and expressed in *E. coli*. *E. coli*-expressing cells were used to compare stress tolerance under stress conditions. Moreover, functional analysis and characterization of *Halothece* GSTs were performed. Results obtained in this study would provide insights into molecular, cellular mechanisms and physiological importance of GSTs. Our results would also contribute to further understanding of the GSTs having several implications in living organisms. Lastly, these enzymes might be applied in biotechnology approaches in the future.

# CHAPTER III

# MATERIALS AND METHODS

## 3.1 Instruments

| | |
|---|---|
| Autoclave | Model ES-215, TOMY Digital Biology, Japan |
| Balance | Model PG2002-S, Mettler Toledo, Switzerland |
| Bench-top centrifuge | MSC-6000, Biosan, Malaysia |
| Biological Safety Cabinet | Model MCV-131S, Sanyo, Japan |
| Cell culture plate (96- and 12-well plate) | SPL Life Science, South Korea |
| Centrifuge bottle | Nalgene™, USA |
| Cuvette (plastic) | Spectronic 401, Milton Roy, USA |
| Cuvette (quartz glass) | Starna®, Optiglass Ltd, UK |
| Deep freezer (-80°C) | Model 8620 forma-86C, Thermo Scientific, USA |
| Freezer (-40°C) | Model DW-40L262, Haier, China |
| Freezer (-20°C) | Sanyo, Japan |
| Gel electrophoresis | Model MJ-105, Major Science, USA |
| Gel imaging | Model Gel Doc EZ™, Bio-Rad Laboratory, USA |
| Glass bottle with screw cap | Duran®, Schott, Germany |
| Horizontal laminar flow | Model H-1, Microtech, Thailand |
| Hot air oven | Model UE600, Memmert, Germany |
| Heat Block | Model TT100-DHC, Hercuvan Lab System, Malaysia |
| Incubator | Model ULE800, Memmert, Germany |
| Incubator shaker | Model Innova-4330, New Brunswick Scientific, USA |
| Laboratory glassware | Pyrex, USA |

| | |
|---|---|
| Magnetic stirrer | Model MMS-3000, Biosan, Latvia |
| Micropipette | Eppendorf Research Plus, Eppendorf, Germany |
| Microplate reader | EnSight™, PerkinElmer, USA |
| Nanodrop 2000 UV-Vis Spectrophotometer | Thermo Scientific™, USA |
| Nano-Q spectrophotometer | Optizen Nano-Q, Mecasys, South Korea |
| Orbital shaker | Model TT-20, Hercuvan Lab Systems, Malaysia |
| Petri-dish (90x15 mm) | Biomed, Thailand |
| pH meter | Mettler Toledo, Switzerland |
| Power supply | PowerPac™ HC, Bio-Rad Laboratory, USA |
| Precision balance | Model ME3002, Mettler Toledo, USA |
| Refrigerator (4°C) | Sanyo, Japan |
| Refrigerated centrifuge | Model 5922, Kubota, Japan |
| Refrigerated microcentrifuge | Model 5418-R, Eppendorf, Germany |
| Rocking platform shaker | Mini Rocker, Bio-Rad Laboratories, USA |
| Sodium dodecyl sulfate polyacrylamide-gel electrophoresis | Model MiniPROTEIN-II®, Tetra Cell, Bio-Rad, Laboratories, USA |
| Sonicator | Vibra-Cell™ Ultrasonic Liquid Processors VCX-130, Sonics, USA |
| Spectrophotometer | GENESYS-20, Thermo Fisher Scientific, USA<br>GENESYS-30, Thermo Scientific, USA |
| Thermo-cycler | Model C-1000 Touch™, Bio-Rad Laboratories, USA |
| Semi-dry transfer cell | Model Trans-Blot® SD Cell, Bio-Rad Laboratories, USA |
| Vortex mixer | Model K-550-GE, Scientific Industries, USA |

**3.2 Chemicals**

| | |
|---|---|
| 30% Acrylamide/Bis Solution | Bio-Rad Laboratories, USA |
| 4-Aminoantipyrine | Sigma-Aldrich, USA |
| 2-mercaptoethanol | Sigma-Aldrich, USA |
| 1-Chloro-2,4-dinitrobenzene (CDNB) | Sigma-Aldrich, USA |
| Acetic acid | Merck, Germany |
| Agar powder | Himedia, India |
| Agarose gel | Bio-Rad Laboratories, USA |
| Ammonium persulfate | Merck, Germany |
| Ampicillin | Amresco, USA |
| Antibody raised against 6-histidine | R&D system, USA |
| Antibody raised against mouse-IgG HRP-conjugated | New England Biolabs, USA |
| Bacto® tryptone | Merck, Germany |
| Bio-Rad protein assay (dry reagent-concentrated) | Bio-Rad Laboratories, USA |
| Boric acid | Merck, Germany |
| Bovine Serum Albumin (BSA) | New England Biolabs, USA |
| Calcium chloride | Merck, Germany |
| Citric acid | Merck, Germany |
| Cobalt (II) nitrate | Ajax Finechem, Australia |
| Coomassie® brilliant blue R-250 | PanReac AppliChem, Germany |
| Copper (II) sulfate | Ajax Finechem, Australia |
| Diethylpyrocarbonate (DEPC) | Amresco, USA |
| Disodium hydrogen phosphate ($Na_2HPO_4$) | Ajax Finechem, Australia |
| Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$) | Ajax Finechem, Australia |
| Disodium ethylenediamine tetraacetate (EDTA: $C_{10}H_{14}N_2O_8Na_2 \cdot 2H_2O$) | Amresco, USA |
| Ethanol | Merck, Germany |
| Ferric ammonium citrate | Merck, Germany |

| | |
|---|---|
| Glycerol | Merck, Germany |
| Glycine | Ajax Finechem, Australia |
| Hydrochloric acid (HCl) | Merck, Germany |
| Hydrogen peroxide ($H_2O_2$) | Merck, Germany |
| Isopropyl β-D-1-thiogalactopyranoside (IPTG) | Sigma-Aldrich, USA |
| Magnesium sulfate | Merck, Germany |
| Manganese (II) chloride | Ajax Finechem, Australia |
| Methanol | Merck, Germany |
| Potassium chloride | Merck, Germany |
| Reduced Glutathione (GSH) | Sigma-Aldrich, USA |
| Saturated Phenol | Amresco, USA |
| Skim milk | Himedia, India |
| Sodium carbonate | Merck, Germany |
| Sodium chloride | Ajax Finechem, Australia |
| Sodium lauryl sulfate | Ajax Finechem, Australia |
| Sodium molybdate | Carlo Erba, Italy |
| Sodium nitrate | Merck, Germany |
| SYBR® safe DNA gel stain | Invitrogen, USA |
| Tetramethylethylenediamine (TEMED) | Bio-Rad Laboratories, USA |
| Trizma base (2-amino-2-(hydroxymethyl)-1,3-propanediol) | Sigma, USA |
| Tween 20 | Merck, Germany |
| Yeast extract powder | Himedia, India |
| Zinc sulfate | Ajax Finechem, Australia |

**3.3 Enzymes**

| | |
|---|---|
| *Nde*I | New England Biolabs, USA |
| *BamH*I | New England Biolabs, USA |
| *Xho*I | New England Biolabs, USA |
| *Taq* DNA polymerase | New England Biolabs, USA |

| | |
|---|---|
| KOD-Fx-Neo (KOD polymerase) | Toyobo, Japan |
| T4 DNA ligase | Takara, Japan |
| RNase | New England Biolabs, USA |

**3.4 Membranes**

| | |
|---|---|
| Nitrocellulose membrane | Merck, Germany |
| Polyvinylidene difluoride (PVDF) membrane | Millipore corporation, USA |

**3.5 Commercial kits**

| | |
|---|---|
| DNeasy® Plant Mini Kit | Qiagen, Germany |
| GenepHlow™ Gel/PCR Kit | Geneaid, Taiwan |
| HiYieldTM Plasmid Mini Kit | RBC Bioscience, Taiwan |
| Horseradish Peroxidase Conjugate Substrate Kit | Bio-Rad Laboratories, USA |
| His-trap™ Affinity Column | GE-healthcare, USA |

### 3.6 Microorganisms and plasmids

**Table 3** Microorganisms and plasmids used in this study

| Strains and plasmids | Descriptions | Sources/references |
|---|---|---|
| ***Halothece* sp. PCC7418** | Halophilic cyanobacterium | This study |
| ***E. coli* DH5a** | F⁻ j80*lacZ*DM15 D(*lac*ZYA-argF) U169 *rec*A1 *end*A1 *hsd*R17($r_K^-$, $m_K^+$) *pho*A *sup*E44 l⁻ *thi*1 *gyr*A96 *rel*A1 | Invitrogen, USA |
| ***E. coli* BL21 (DE3)** | F⁻ *omp*T *hsd*$S_B$ ($r_B^-$, $m_B^-$) *gal dcm* (DE3) | Invitrogen, USA |
| ***E. coli* ATCC8739** | *E. coli* wild type | Microbial Culture Collection, Department of Microbiology, Faculty of Science, Chulalongkorn University |
| **pET15b** | Cloning and expression vector | Invitrogen, USA |
| **pGEX6P-1** | Expression vector | GE healthcare, USA |
| **pET15b_0647** | 552 bp *PCC7418_0647* fragment cloned into pET1b | This study |
| **pET15b_0729** | 1,200 bp *PCC7418_0729* fragment cloned into pET1b | This study |
| **pET15b_1478** | 561 bp *PCC7418_1478* fragment cloned into pET1b | This study |
| **pET15b_3557** | 801 bp *PCC7418_3557* fragment cloned into pET1b | This study |

### 3.7 Primers

**Table 4** Primers for cloning of *Halothece* GSTs

| Primers | Sequences (5' to 3') |
|---|---|
| **GST0647pET15b_NdeI (F)** | TAATAAATAACACATATGCTTAAACTATATGGTGCAACC |
| **GST0647pET15b_BamHI (R)** | CAGAAACTTGATGGATCCTTAGAAGCCCATTC |
| **GST0729pET15b_NdeI (F)** | AAGTTAAGTATTCATATGCAGGCACTGAGTTGGG |
| **GST0729pET15b_BamHI (R)** | TCTTCTCTGCGAGGATCCTCAAACTTTTGCAAAA |
| **GST1478pET15b_NdeI (F)** | AGACCAATGGTACATATGAAACTTTATTATCTTCCGT |
| **GST1478pET15b_BamHI (R)** | TCAGATAATTTTGGATCCTCACGGGGGTTTCTTT |
| **GST3557pET15b_NdeI (F)** | AGCGAATGCACTCATATGTTAGAACTTTATCAAT |
| **GST3557pET15b_BamHI (R)** | AACTAAATTAAGGGATCCTTACTCAATTTCAATAGAAC |

**Table 5** Primers for DNA sequencing and colony PCR

| Primers | Sequences (5' to 3') |
|---|---|
| **T7-terminator** | GCTAGTTATTGCTCAGCGG |
| **T7-promoter** | TAATACGACTCACTATAGGG |
| **PCR_GST0647-Forward** | GCGATTGAAGATAATGGCT |
| **PCR_GST0647-Reverse** | ACATTCTGGGCATATAAGCT |
| **PCR_GST0729-Forward** | GTCCTTATTTCCGAGACAGC |
| **PCR_GST0729-Reverse** | ACATCAGGTAAACCTAGCCA |
| **PCR_GST1478-Forward** | TTTAGCCGATCAATATCCTG |
| **PCR_GST1478-Reverse** | ACCTGTAATAACATCAGCAG |
| **PCR_GST3557-Forward** | CTCAAGCAAGATTTAGAGGC |
| **PCR_GST3557-Reverse** | TTTCAATAGAACTGGGTGCA |

### 3.8 Culture conditions

*Halothece* sp. PCC7418 was typically cultured in BG-11 medium + Turk solution with 0.5 M NaCl on a shaker, under continuous light (30-50 μmol $m^{-2}$ $s^{-1}$) at 30 ± 2°C (Waditee-Sirisattha et al., 2014). *E. coli* strains DH5α and *E. coli* BL21 were used as cloning and expressing host cells, respectively. These two *E. coli* strains were cultured in Luria-Bertani (LB) broth or LB agar on a shaker (110 rpm) at 37°C. When growing *E. coli* strains harboring plasmids, ampicillin was supplied (a final concentration of 75 μg/ml). The absorbance at 600 nm or 730 nm were measured to determine the growth of *E. coli* and cyanobacteria, respectively.

### 3.9 Bioinformatics and phylogenetic analysis

#### 3.9.1 Basic features and putative functions of genes

Putative GSTs encoding genes in cyanobacterium *Halothece* sp. PCC7418 were searched and analyzed using public database, in Kyoto Encyclopedia of Genes and Genomes (KEGG) (www.genome.jp/kegg/). The information of nucleotide sequences, amino acid sequences, pI and theoretical mass were also obtained from the KEGG database. Gene ontology (GO) function of the *Halothece* GSTs were defined by Uniprot database (http://www.uniprot.org/).



#### 3.9.2 Domain architecture

The structural regions (protein domains) of *Halothece* GSTs were identified and generated the map by Expasy Prosite (https://prosite.expasy.org/), using UniProtKB accession number of the GSTs as a query.

#### 3.9.3 Phylogenetic analysis

The amino acid sequences *Halothece* GSTs and other 67 orthologs from nine extremophilic cyanobacteria were obtained from KEGG database. These include *Thermosynechococcus elongatus, Euhalothece natronophila, Gloeocapsa* sp. PCC7428, *Pleurocapsa* sp. PCC7327, *Prochlorococcus marinus, Dactylococcopsis salina, Rivularia* sp. PCC7116, *Halomicronema hongdechloris* and *Acaryochloris marina*. In addition, GST from *Escherichia coli* K12 (JW1627) was used as a

representative member of mesophilic bacterial GST. The alignment and phylogenetic tree reconstructions were conducted using MEGA7 program (https://mega.software.informer.com/7.0/) (Kumar et al., 2016). The alignment was performed with MUSCLE method (Edgar, 2004). The tree was constructed using neighbor-joining method (Saitou & Nei, 1987). The test of reliability was performed by bootstrap method with 300 replicates.

### 3.9.4 Three-dimension model analysis

The amino acid sequences of *Halothece* GSTs were used for prediction and generating of three-dimension model via Expasy Swiss-Model (https://swissmodel.expasy.org/interactive). GST from *Escherichia coli* K12 was used as a representative member of mesophilic bacterial GST.

## 3.10 Cloning and expression of *Halothece* GSTs in *E. coli*

### 3.10.1 Cloning of *Halothece* GSTs

Genomic DNA of *Halothece* sp. PCC7418 was extracted by using DNeasy Plant mini-kit (Qiagen, Germany), according to the manufacturer's recommendations. PCR amplification was performed by using KOD-Fx-Neo (Toyobo, Japan) to amplify each *Halothece* GST encoding genes, using specific primer pairs as described in Table 3. These primers were designed using Perl Primer (http://www.perlprimer. sourceforge.net). In all cases, a specific forward primer contains a NdeI restriction site, and a specific reverse primer contains a BamHI restriction site as well as 12 base pairs of upstream and downstream regions of *Halothece* GST genes. Then, the amplified DNA fragment for each GST gene was digested and ligated into pET15b at the corresponding sites, generating the constructs pET15b_0647, pET15b _0729, pET15b _1478 and pET15b _3557, respectively. Each recombinant plasmid was transformed into *E. coli* DH5$\alpha$. After that, the recombinant plasmids were prepared using HiYield$^{TM}$ Plasmid Mini Kit. After verification by nucleotide sequencing, each recombinant plasmid was then transformed into *E. coli* BL21. Hereafter, the *E. coli* BL21 containing pET15b_0647, pET15b _0729, pET15b _1478, pET15b _3557, empty

vector pET15b and empty vector pGEX6P-1 were designated as GST_0647, GST_0729, GST_1478, GST_3557, pET15b and pGEX6P-1, respectively.

### 3.10.2 Protein expression analysis of *Halothece* GSTs

Protein expression was carried out following manufacterer's instructions. Briefly, four recombinant *E. coli* BL21 cells containing each recombinant plasmid (pET15b_0647, pET15b_0729, pET15b_1478 or pET15b_3557) was cultured in LB liquid medium at 37°C until $OD_{600}$ reached to 0.8. Then, protein expression was induced by adding IPTG at a final concentration of 0.5 mM. After incubation for overnight, crude proteins were extracted and prepared by sonication using Vibra-cells™ sonicator. Crude extracts and supernatants from recombinant *E. coli* cells were separately determined for protein concentrations by Bradford assay. Furthermore, SDS-PAGE analysis and Western blotting were carried out according to standard protocols (Sambrook, 2001) to analyze protein expression.

## 3.11 In vivo stress tolerance of GST expressing cells

### 3.11.1 Salt stress treatment

Four recombinant *E. coli* BL21, cells carrying *Halothece* GSTs (GST_0647, GST_0729, GST1478 and GST_3557) were subjected to salt stress and survival rate was compared. In addition, pGEX6P-1, which contains GST encoding gene from *Schistosoma japonicum*, and pET15b (empty vector control) were used as control groups. Expressing cells were cultured in LB media at 37°C for overnight. Next day, the expressing cells were transferred into fresh LB media containing 0.7M NaCl, using 5% inoculum. The initial $OD_{600}$ for all cultures were set approximately 0.3-0.4. There were two sets of treatments, first one was without IPTG added, while another set IPTG was added (at a final concentration 0.5 mM) in the last step. Each set was performed in triplicate. The cells were cultured on shaker at 37°C for 24 hours. After that, all treated cells were 10-fold serially diluted (from $10^{-1}$ to $10^{-6}$). Thereafter, 2.5 µl of each diluted cell were dropped onto LB agar plate. After incubation at 37°C for overnight, stress tolerance was scored by assessing growth or lack of growth. Viable cells were calculated from colony forming unit (CFU).

### 3.11.2 $IC_{50}$ determination of $H_2O_2$ for *E. coli* expressing cells

The $IC_{50}$ value for *E. coli* BL21 upon $H_2O_2$ treatment was determined. In brief, pET15b was cultured in LB medium at 37°C until $OD_{600}$ reached 0.6-0.8. Then, $H_2O_2$ was added into cell cultures at final concentration of 0, 1, 4, 8, 10, 12, 14 and 16 mM, respectively. The cell growth was measured via $OD_{600}$ at 24 and 42 hours. The data were transformed into percentage of viable cells, and then plotted on graph to determine the $IC_{50}$ value.

### 3.11.3 Oxidative stress treatment

Four recombinant *E. coli* BL21 cells carrying *Halothece* GSTs (GST_0647, GST_0729, GST1478 and GST_3557) were subjected to oxidative stress-induced by $H_2O_2$ and survival rate was compared. The same as conducting with salt stress, pET15b and pGEX6P-1 were sued as control groups. All recombinant cells were cultured in LB at 37°C for overnight. Next day, the expressing cells were transferred into fresh LB media with $H_2O_2$ (at a final concentration correspond to $IC_{50}$). The initial $OD_{600}$ for all cultures were set approximately 0.3-0.4. There were two sets of treatments, first one was without IPTG added, while another set IPTG was added (a final concentration of 0.5 mM) in the last step. Each set was performed in triplicate. The cells were cultured on shaker at 37°C for 48 hours. Finally, all treated cells were 10-fold serially diluted (from $10^{-1}$ to $10^{-6}$). Thereafter, 2.5 µl of each diluted cell were dropped onto LB agar plate. After incubation at 37°C overnight, stress tolerance was scored by assessing growth or lack of growth. Viable cells were calculated from colony forming unit (CFU).

### 3.11.4 Metal stress treatment

The recombinant *E. coli* BL21 cells carrying *Halothece* GST which was conferred the best ability to survive under salt and oxidative stress was additionally tested under metal associated stress. The pET15b and pGEX6P-1 vectors were used as control groups. Moreover, *E. coli* ATCC8739 (wild type) was also used as negative control. All cells were cultured in LB media on shaker at 37°C until $OD_{600}$ reached 0.64±0.05. Thereafter, the cells were spread on LB agar plate. LB for *E. coli* wild type

is not supplied with antibiotic. Next, the copper and silver disc (5 mm dimension) were placed onto agar plates containing bacterial culture. The pound paper disc was placed as a negative control. After overnight incubation at 37°C, the inhibition zone surrounding metal discs and pound paper disc were measured. The smaller inhibition zone refers to higher tolerance of the cells.

### 3.12 Extraction and purification of *Halothece* GSTs

#### 3.12.1 Crude protein preparation

According to stress tolerance determination in section 3, the *E. coli* BL21 expressing *Halothece* GST in which conferred the best stress tolerance was selected for further purification and functional characterization. For crude protein preparation, the expressing cells were cultured on shaker at 37°C until $OD_{600}$ was reached to 0.6-0.8. Then, the IPTG (at a final concentration of 0.5 mM) was added, and continued culturing at 30°C, for 6 hours. Thereafter, the cells were harvested by centrifugation at 8,000 rpm, 4°C for 10 minutes. The crude proteins were extracted by sonication. Supernatant solutions were collected by centrifugation at 12,000 rpm, 4°C for 5 minutes. The crude proteins and supernatant solutions were kept at 4°C until analysis.



#### 3.12.2 Purification of recombinant *Halothece* GSTs

Supernatant solutions were prepared and subjected to affinity purification by using His-trap™ His-tag resin. Batch purification was performed with initial total crude protein of 7 mg. Tris-Cl buffer containing imidazole (in a range of 200-500 mM) was used to elute 6-His-tag-*Halothece* GST fusion proteins. Purified *Halothece* GST was desalted to remove imidazole and salts by dialysis using cellophane membrane bags (molecular weight cut off 3000 Da). Purified recombinant proteins was confirmed the purity by SDS-PAGE and Western blot analysis, respectively. Protein concentration was measured by Bradford assay.

### 3.13 Functional characterization of *Halothece* GSTs

#### 3.13.1 Glutathione S-transferase activity assay

The purified *Halothece* GST (2.5 μg) was used for standard glutathione S-transferase activity assay. Two substrates were used in this reaction, reduced glutathione (GSH) and 1-chloro-2, 4-dinitrobenzene (CDNB). The forming of conjugated product, S-(2,4-dinitrophenyl) glutathione, was determined using a spectrophotometer at wavelength 340 nm ($A_{340}$) for 5 minutes. One unit of enzyme was defined as the conjugation of 1.0 μmole of CDNB with GSH per minute at 25°C (Simons & Vander Jagt, 1977). The optimization of buffer and pH was performed using excess substrates (1.3 mM GSH and 0.5 mM CDNB) in tested buffers and pH range, including Tris-Cl buffer (pH 6.4-10.5), sodium phosphates buffer (pH 5.5-8.5) and MES buffer (pH 5.5-7.5).

In order to examine the steady-state kinetic parameters of the *Halothece* GSTs, the apparent $K_m$ values for GSH and CDNB were separately determined by varying the concentration in one of them and keeping constant the other. The experiments were performed under optimal pH and buffer in triplicate.

#### 3.13.2 Effect of salt on GST activity

GST_3557 was also determined for glutathione s-transferase activity in optimal buffer with 0-2 M NaCl to investigate the effect of salt. The enzyme and substrates used in experiment are the same as described in chapter 3.13.3.



#### 3.13.3 Peroxidase activity assay

Peroxidase activity assay was performed to observe additional function of *Halothece* GST. The assay includes 12 mM phenol (350 μl), 0.5 mM 4-aminoantipyrine (100 μl), 0.7 mM $H_2O_2$ (160 μl) as substrates, purified GST (25 μg) and phosphates buffer pH 7.5 (adjusted volume to 1,000 μl). At first, all substrates are colorless. If the enzyme exhibit peroxidase activity, $H_2O_2$ will be catalyzed to react with phenol and 4-aminoantipyrine (4-APP). Quinoneimine, as known as the formed pink-product, was measured via spectrophotometry at wavelength 504 nm ($A_{505}$) for

3-5 minutes (Fernando & Soysa, 2015). Horse-radish peroxidase (HRP) was used as a positive control.

# CHAPTER IV
# RESULTS AND DISCUSSIONS

## 4.1 Bioinformatics analysis

Based on genome-based information on Kyoto Encyclopedia of Genes and Genomes (KEGG), there are four putative GST encoding genes in *Halothece* sp. PCC7418. Features and physicochemical properties of *Halothece* GSTs are summarized in Table 6. The nucleotide and amino acid sequences of *Halothece* GSTs were additionally provided in the appendices.

Four *Halothece* GSTs have different features and characteristics, such as theoretical molecular weight and pI. Among four *Halothece* GSTs, PCC7418_0647 and PCC7418_1478 comprised of similar number of nucleotides and amino acid residues, as well as theoretical molecular masses. In contrary, PCC7418_0729 and PCC7418_3557 are distinct from the other two *Halothece* GSTs. PCC7418_0729 is the largest *Halothece* GST with theoretical molecular mass of 46.34 kDa. This protein size is considerably large compared with common GSTs (25-30 kDa) (Shehu et al., 2019). PCC7418_3557 had the lowest pI (4.6) compared to other *Halothece* GSTs which the actual values were 5.0-5.4. It was evident that cytosolic GST usually comprises of two conserved domains, N- and C domain. All *Halothece* GSTs consisted of N domain with approximately 78-80 amino acid residues. However, two *Halothece* GSTs including PCC7418_0729 and PCC7418_3557 lack of C domain. Some public databases suggest that they comprised of low complexity region with feature similar to C domain. The H-site possibly presents in this region. Special features of these *Halothece* GSTs might related to functions under stress environment.

The gene ontology (GO) function suggests that the molecular function of all *Halothece* GSTs involved in transferase activity. All *Halothece* GSTs were not associated with biological process as well as cellular components (Table 6).

**Table 6** Bioinformatics analysis of *Halothece* GSTs

| | | GST_0647 | GST_0729 | GST_1478 | GST_3557 |
|---|---|---|---|---|---|
| KEGG gene accessing number | | PCC7418_0647 | PCC7418_0729 | PCC7418_1478 | PCC7418_3557 |
| Uniprot protein accessing number | | K9Y8X8 | K9Y7Y6 | K9Y7Y6 | K9YGQ3 |
| Nucleotide (base pairs) | | 552 | 1,200 | 561 | 801 |
| Amino acid (residues) | | 183 | 399 | 186 | 266 |
| Theoretical molecular weight (kDa) | | 20.86 | 46.34 | 21.58 | 29.46 |
| Theoretical pI | | 5.05 | 5.48 | 5.44 | 4.60 |
| N-domain amino acid (residues) | | 80 | 82 | 78 | 78 |
| C-domain amino acid (residues) | | 102 | - | 103 | - |
| Low complexity regions/region features (residues) | | - | 28 | - | 22 |
| GO function | Molecular functions | Transferase activity | Transferase activity | Transferase activity | Transferase activity |
| | Biological processes | Not involve | Not involve | Not involve | Not involve |
| | Cellular components | Not involve | Not involve | Not involve | Not involve |

**4.2 Phylogenetic analysis and domain architecture**

A phylogenetic tree of four putative GST from *Halothece* sp. PCC7418 and other 61 orthologs was constructed (Figure 4). The *E. coli* K12 GST was included as a representative member of mesophilic bacteria. The tree suggests that four *Halothece* GSTs were diverse and distributed in different clades. For example, PCC7418_0647 shared the highest homology with GST from cyanobacterium *Dactylococcopsis salina* (Dacsa_2391), with 18% amino acid sequence similarity. PCC7418_0729 also shared the highest homology with *D. salina* GST (Dacsa_2853), but with approximately 77% sequence similarity. In contrary, PCC7418_1478 shared the highest homology with GST from cyanobacterium *Euhalothece natronophila* (FRE64_15440), with 65% sequence similarity. Last one, PCC7418_3557 shared the highest homology with *D. salina* GST (Dacsa_1405), with 87% amino acid similarity. Both *D. salina* and *Euhalothece* sp. are the halophilic cyanobacterium as same as *Halothece* sp. PCC7418 (Walsby et al., 1983; Yang et al., 2020). Moreover, PCC7418_0647 lied on the same clade with GST expressed from cyanobacterium *Thermosynechococcus*

*elongatus* (tlr0207), but the sequence similarity between these GSTs is only 17%. Tlr0207 was recently classified as novel Chi-class GST (Wiktelius & Stenberg, 2007). In addition, PCC7418_3557 also shared homology to GST expressed from cyanobacterium *Prochlorococcus marinus* (Pro_0130), with 37% amino acid similarity. This GST was classified as a Zeta-class GST, which can be generally found in various organisms (Dufresne et al., 2003; Perperopoulou et al., 2018).

Phylogenetic tree is one of informative tools for classification of GST. The cytosolic GSTs within the same class should share amino acid sequence identity more than 40% (Ochi, 2017). Thus, these results only can be proposed that PCC7418_0647 and PCC7418_3557 are closest to Chi-class and Zeta-class GST homolog, respectively. These two *Halothece* GSTs might contain some characteristics or abilities similar to the closet class, but this cannot be clearly classified into Chi- or Zeta class. In contrary, PCC7418_0729 and PCC7418_1478 had no homology to any identified GST classes. For further classification of these GSTs, substrates specificity, kinetics, and protein-protein interaction need to be clarified (Pandey et al., 2017b).

Domain architecture was analyzed using expert curation in UniProtKB/Swiss-Prot in which were defined by the InterPro resource, PROSITE, Pfam. It revealed that cyanobacterial GSTs were ranged from 93 to 416 amino acid residues with typically organized by N- and C domains (Figure 4). The number of amino acid residues for N- and C domain are denoted in both Figure 4 and Table 6. These models suggest that almost all GSTs consisted of N-domain with approximately 75-85 amino acid residues, except GSTs from *Prochlorococcus marinus* (Pro_0250), *Euhalothece natronophila* (FRE64_11270), *Rivularia* sp. (Riv7116_6393) and *Pleurocapsa* sp. (Ple7327_1183). These four GSTs lacked N domain; however, they consisted of low complexity regions. These results also revealed that PCC7418_0729 and PCC7418_3557 lack of C domain. They consisted of low complexity region that involved in some features, similar to the C domain. The plausible reason is PCC7418_0729 and PCC7418_3557 likely contained a region of distinct amino acid residues at C terminus which is different from other GSTs but suitable for function under unique stress condition. Apart from PCC7418_0729 and PCC7418_3557, the domain architecture model also suggests that some GSTs from other cyanobacteria,

such as *Euhalothece natronophila* (FRE64_03145)*, Rivularia* sp. (Riv7116_2857 and Riv7116_3320) and *Pleurocapsa* sp. (Ple7327_2157), all lack of C domain but contain other low complexity regions too. In addition, phylogenetic analysis shown that these cyanobacterial GSTs were in the same clade of *Halothece* GST_0729 or GST_3557 (Figure 4).

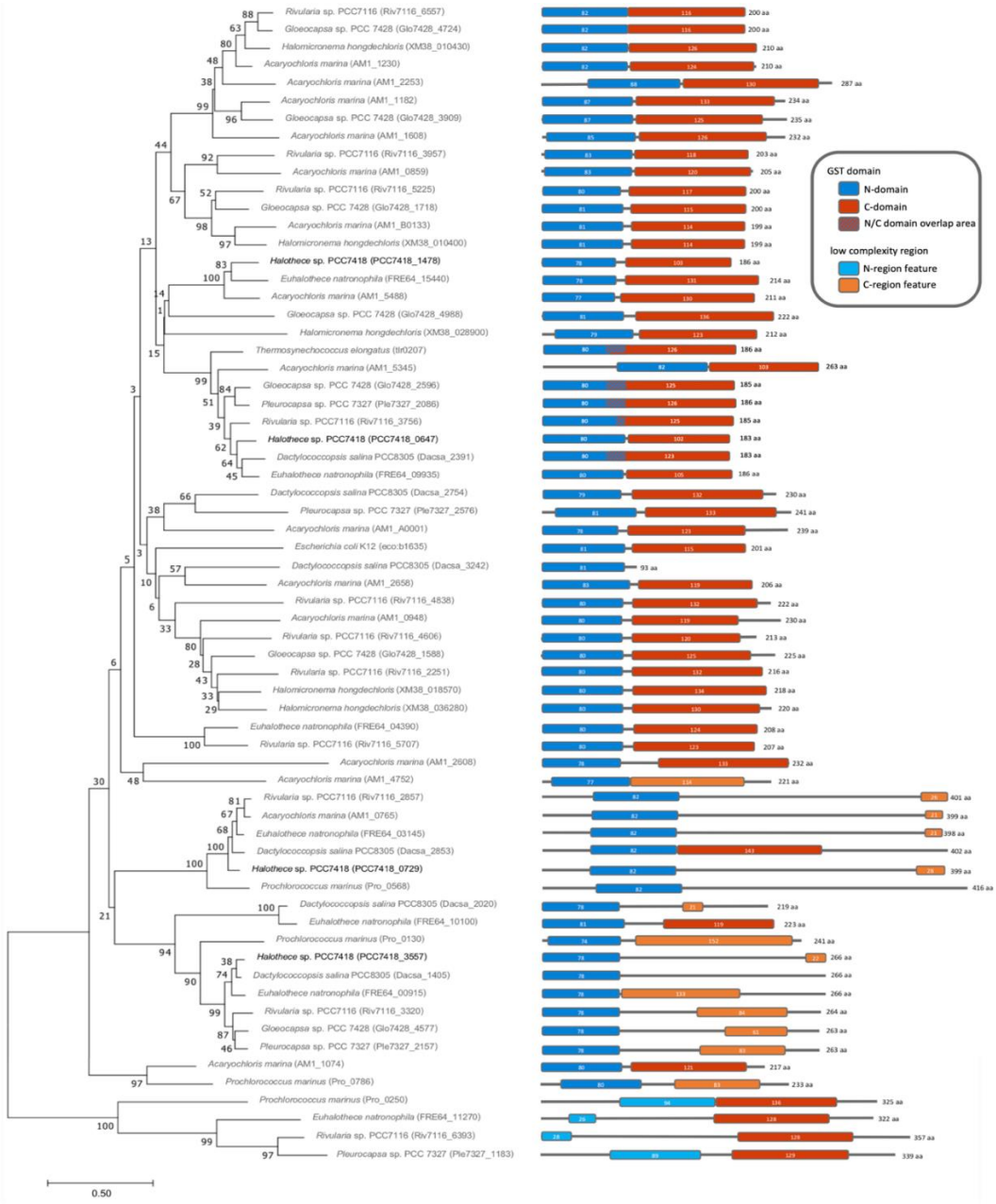


**Figure 4** Phylogenetic tree and domain architecture of *Halothece* GSTs and cyanobacterial orthologs. The tree was constructed with Neighbor-Joining method, with the 300 replicates bootstrap. The tree was presented with a specific epithet together with KEGG gene accession number in bracket. The scale bar, represent evolutionary distance, comprises 0.5 expected changes per amino acid site. Bootstrap probabilities are shown at the nodes.

### 4.3 Three-dimension model analysis

The three-dimension models of four *Halothece* GSTs, the *E. coli* K12 (JW1627) GST and *Schistosoma japonicum* Mu-class GST (expressed from recombinant cell containing pGEX6P-1) were generated using Expasy Swiss-Model (Figure 5). The models suggest that all GSTs are formed in homodimer. It should be noted that *Halothece* GSTs possess some different structure and folding compared to *E. coli* GST and *S. japonicum* (pGEX6P-1) GST. In addition, the folding of each *Halothece* GSTs was also diverse. Especially GST_3557 consisted of helices more than other GSTs. These models implied that a part of *Halothece* GST structure is evolutionary modified and might be suitable for some unique functions under stress or adverse conditions. These features were not found in *E. coli* and *S. japonicum* GSTs.

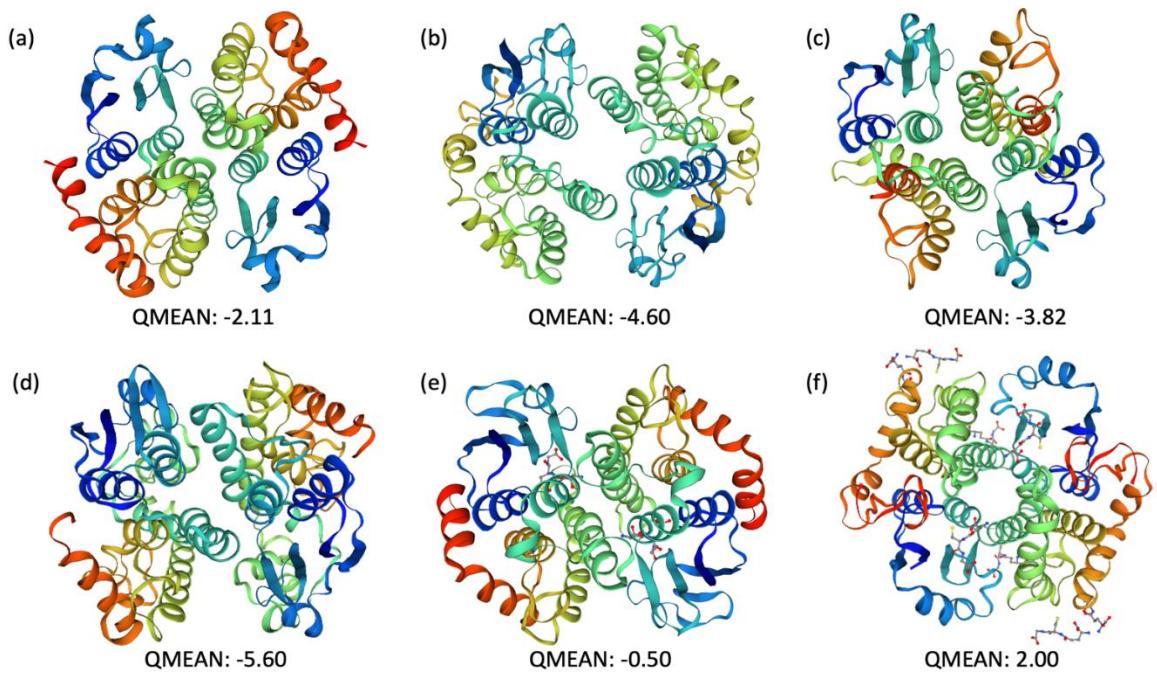


**Figure 5** The three-dimension model of *Halothece* GSTs: (a) GST_0647, (b) GST_0729, (c) GST_1478, (d) GST_3557, (e) *E. coli* GST (JW1627), and (f) *S. japonicum* GST expressed from pGEX6P-1 vector. The models were constructed using Expasy Swiss-Model. The Q-mean value of each model was shown under the model.

**4.4 Cloning and expression of *Halothece* GSTs in *E. coli***

In previous study, the *Halothece* GSTs were cloned and expressed into pColdI system; however, recombinant proteins were not obtained, implicating pColdI system was not suitable (Samun, 2019). In this study, the pET expression system featuring the T7 promoter was used to express four *Halothece* GSTs. Recombinant PCC7418_0647, PCC7418_0729, PCC7418_1478 and PCC7418_3557 were successfully produced in this expression system. This expressing vector contained 6-Histidine tag sequence, thus allowing the target fusion protein can be purified by His-tag affinity chromatography. Also, this feature facilitates protein expression by Western blotting.

The recombinant plasmids were firstly transformed into *E. coli* DH5$\alpha$. Then, nucleotide sequencing of all full-length gene was performed for verification. The nucleotide sequence of each *Halothece* GST was provided in the appendices. We found the perfect match of pET15b_0647, pET15b_0729 and pET15b_1478 with sequence data of KEGG. In case of pET15b_3557, one nucleotide was found to be mismatched from the putative sequence (C446A). Multiple alignment of pET15_3557 and six closet cyanobacterial GST orthologs (including *D. salina* (Dacsa_1405_, *Euhalothece natronophila* (FRE64_00915), *Rivularia* sp. (Riv7116_3220), *Gloeocapsa* sp. (Glo7428_4577) and *Pleurocapsa* sp. (Ple7327_2157)) suggested that the substitution site in this case (amino acid residue 49) is not a conserved residue (the appendices). Thus, the amino acid substitution in pET15b_3557 could not affect the protein folding or biochemical characteristics.



The recombinant plasmids (pET15b_0647, pET15b_0729, pET15b_1478 and pET15b_3557) were further transformed into expressing cells, *E. coli* BL21. Colony PCR was performed to confirm the successful of transformation (Figure 6). Thereafter, these transformants were used for recombinant protein expression.

Protein expression was induced by IPTG at a final concentration of 0.5 mM, in each recombinant *E. coli* culture during the exponential growth phase ($OD_{600}$ was reached ≈ 0.6-0.7). Target *Halothece* GSTs proteins were observed by SDS-PAGE analysis (Figure 7). All the proteins were highly expressed but mostly as inclusion forms, except for GST_0647. Therefore, the temperature and culture conditions were further optimized to increase soluble forms of GSTs (GST_0729, GST_1478 and

GST_3557). The optimization was performed by varying temperature (37°C, 30°C and 16°C) together with period of induction (Figure 8). Our results revealed that incubation at 30°C for six hours was the most appropriate to obtain soluble fraction. Lastly, we used this condition for preparation of recombinant proteins.

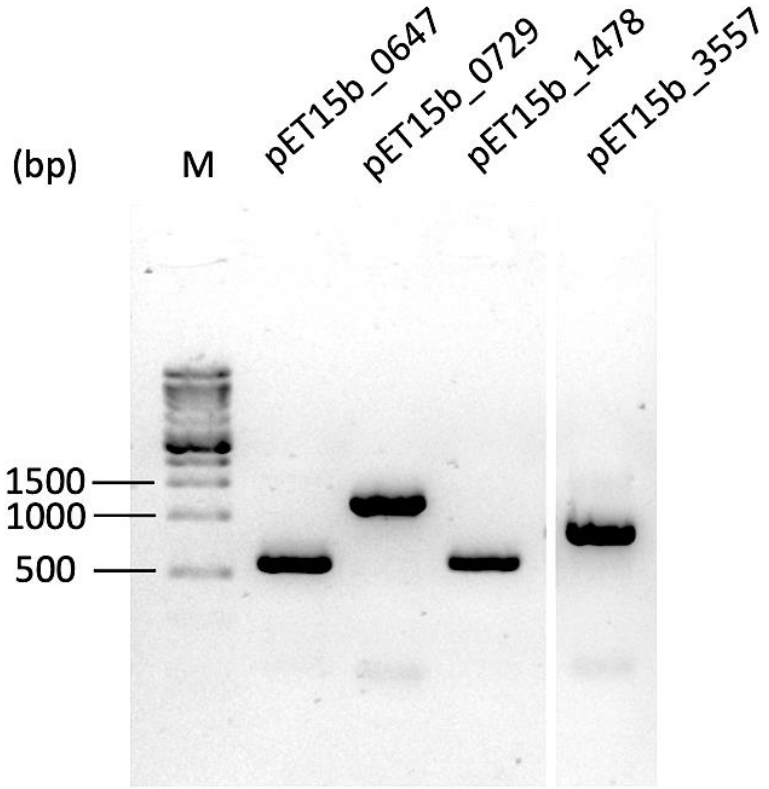




**Figure 6** Colony PCR to confirm successful transformation of pET15b_0647, pET15b_0729, pET15b_1478 and pET15b_3557 plasmids in *E. coli* BL21 cells. This PCR was performed using specific primer pairs for each GST gene.

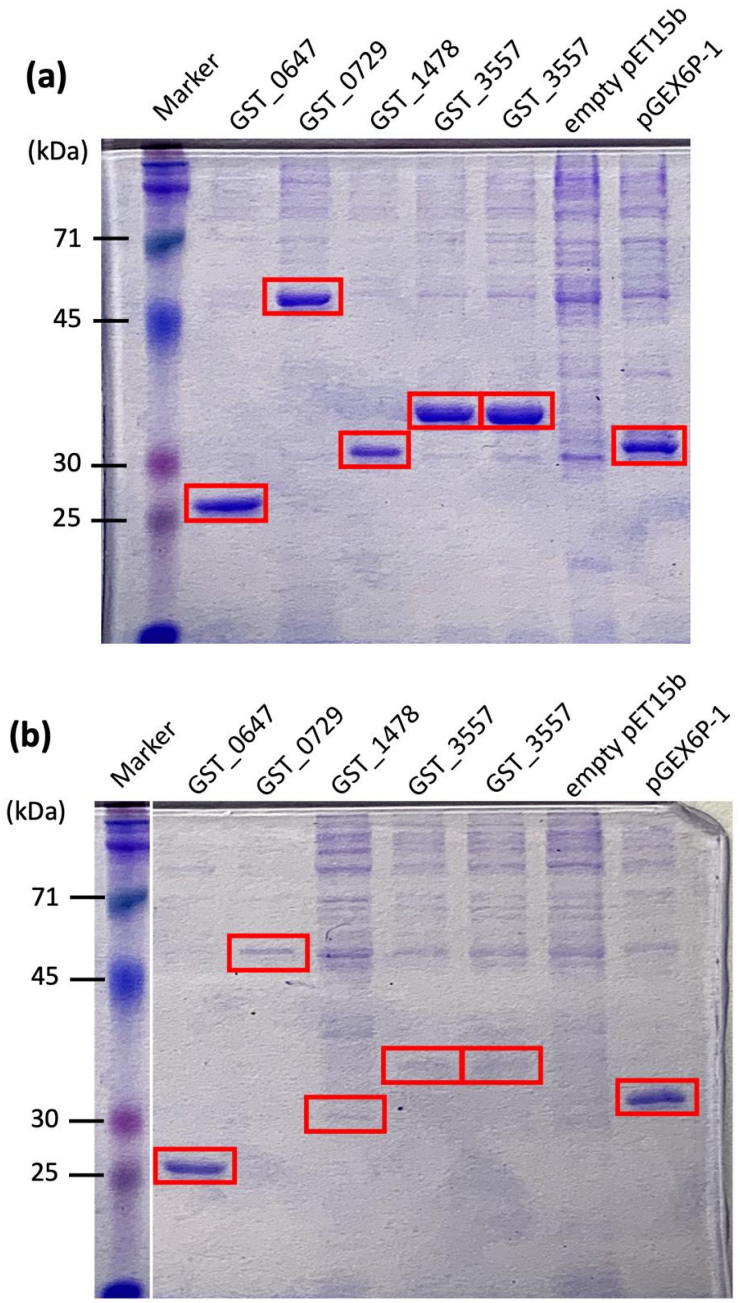


**Figure 7** SDS-PAGE analysis of total expressed proteins from the *E. coli* BL21 expressing GST genes obtained from (a) crude lysates and (b) supernatants, using 10% SDS-PAGE stained with Coomassie-brilliant blue R-250. Protein expression was induced by adding IPTG at a final concentration of 0.5 mM and culturing for 18 hours.

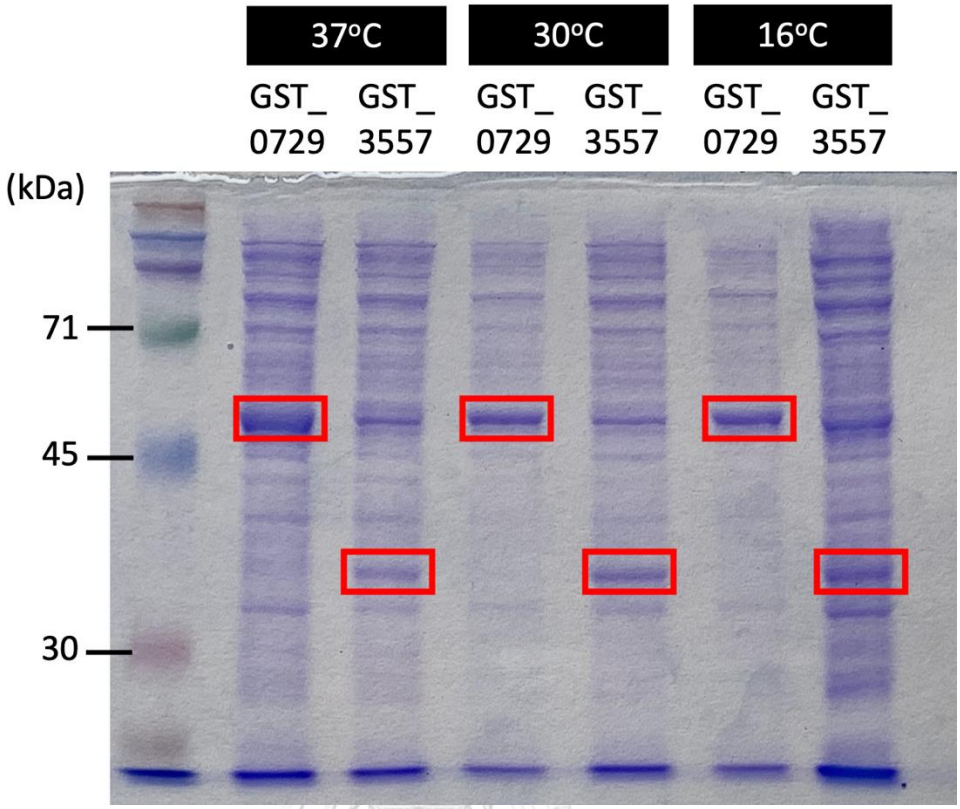


**Figure 8** Optimization to increase GST_0729 and GST_3557 expression levels in soluble forms. IPTG was used at a final concentration of 0.5 mM. After IPTG induction, cells were cultured by varying temperature for six hours under shaking condition. Soluble proteins were obtained by sonication and centrifugation. Protein expression was analyzed by 10% SDS-PAGE stained with Coomassie-brilliant blue R-250.

### 4.5 *In vivo* stress tolerance of GST recombinants

#### 4.5.1 Salt Stress

Four expressing cells (GST_0674, GST_0729, GST_1478 and GST_3557) and two control cells (pET15b and pGEX6P-1) were cultured under salt stress (0.7 M NaCl) for 24 hours. The experiment was performed in two sets, with or without IPTG. Then, the stressed cells were 10-fold serially diluted ($10^{-1}$ to $10^{-6}$) and dropped onto LB agar plate. The survival cells were scored after 18 hours of incubation. The result revealed that the viable cells for GST_0647, GST_0729, GST_1478 and GST_3557 were significantly higher than those of pET15b and pGEX6P-1. This result suggested that all *Halothece* GSTs contributed for cellular defensive mechanism against salt stress. Without IPTG adding, GST_3557 exhibited the best performance, evaluating from CFU after subjecting to salt stress. The viable cell count of GST_3557 was also statistically higher than pET15b, approximately 18-folds (Figure 9a). This result is in agree with gene expression analysis demonstrating that PCC7418_3557 was highly up-regulated under salt stress (Kortheerakul, 2019). Thus, GST_3557 is likely to be the most crucial detoxification enzyme amongst four *Halothece* GSTs, and highly responses upon salt stress.

By using independent set, IPTG was included from the fact that pET vector drives gene expression by T7 promoter. For the cell expressing pGEX6P-1 (the *S. japonicum* GST in plasmid), protein was expressed at higher level. Thus, it is reasonable that the expressing cells cultured with IPTG could survive more than that of without IPTG adding. However, we observed that all expressing cells carrying *Halothece* GSTs performed lower survival ability (Figure 9b). Although the growth rate of *E. coli* BL21 under LB without IPTG and LB supplemented with 0.5mM IPTG were similar, suggesting IPTG was not affected the cell growth under non-stress condition (The appendices). We suspected that the IPTG might possibly affect the growth of expressing cells subjected to stress. IPTG is the molecule used to induce the protein expression in plasmid containing T7-promoter. IPTG binds to lac-repressor and allows T7-RNA polymerase to initiate transcription of target gene, next to the T7-promoter. This system is widely used for *E. coli* expression system (Gomes et al., 2020). This substrate is not an innocuous inducer, but in some cases, can indirectly affect the

cell growth (Dvorak et al., 2015). Bacterial growth depends on several factors, such as energy, nutrients and cellular materials, including enzymes and metabolic precursors. However, some of these factors are the limitation (Kempes et al., 2017). Under stress condition, the cells usually need more energy and cellular materials to defense against stress factors and maintain cellular homeostasis (Valentine, 2007). Moreover, a high expression of recombinant proteins increases the demand of energy and cellular materials. Both recombinant proteins expression and cellular stress response at the same time may cause insufficient of cellular energy. Finally, the cell lacks energy for growth and bring to the decreasing of growth rate (Malakar & Venkatesh, 2012). This is one the plausible reasons to explain why the cell count from salt stress treatment supplemented with IPTG resulted in lower viable cell count. In addition, there is another reason to explain this phenomenon. Based on the results in chapter 4.4, most of *Halothece* GSTs were expressed as inclusion forms after induction by IPTG. These forms of proteins cannot be functioned to respond against stress conditions as well. Thus, this might be resulted in the lower survival efficiency of the expressing cells in this case. While in case that IPTG was not added, the recombinant proteins were slightly express in low level, which might not be toxic to the cells and not resulted in inclusion forms.

Under salt-stress treatment together with the presence of IPTG, the clone that conferred the best survival efficiency was GST_0647. This might be the combined effect of salt stress and insufficient cellular energy, caused by IPTG as mentioned above. The result suggested that *Halothece* GSTs are diverse and might play a different role in different condition. However, it is still difficult to explain this phenomenon. Further expression analysis of *Halothece* GSTs encoding genes under IPTG-related energy stress should be performed.

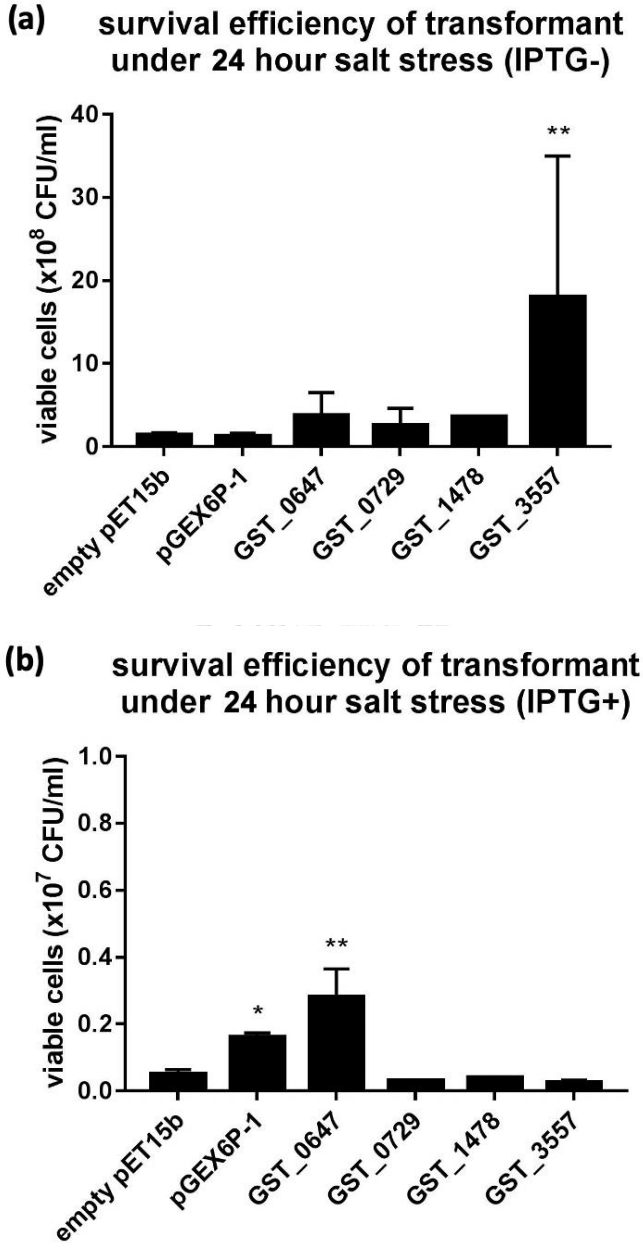


**Figure 9** Survival efficiency of expressing cells containing each recombinant GST gene after treated under salt stress for 24 hours, (a) no IPTG added, and (b) added 0.5mM IPTG. The stars shown significant level, * is sig < 0.05 and ** is sig < 0.01, at 95% confidence level.

### 4.5.2 $IC_{50}$ determination of $H_2O_2$ for *E. coli* expressing cells

The exponential phase expressing cell carrying empty pET15b was cultured in LB media supplemented with $H_2O_2$ varied concentration from 0 to 16 mM for 42 hours. The growth was observed via $OD_{600}$, then calculated to percentage of viable cells. The $IC_{50}$ value was calculated using Graph-pad Prism 7.0 software (Figure 10). The $IC_{50}$ value for *E. coli* expressing cells against $H_2O_2$ was 10.61±1.02 mM (at 24 hours) and 11.10±1.02 mM (at 42 hours). Thus, $IC_{50}$ was used for next experiments.

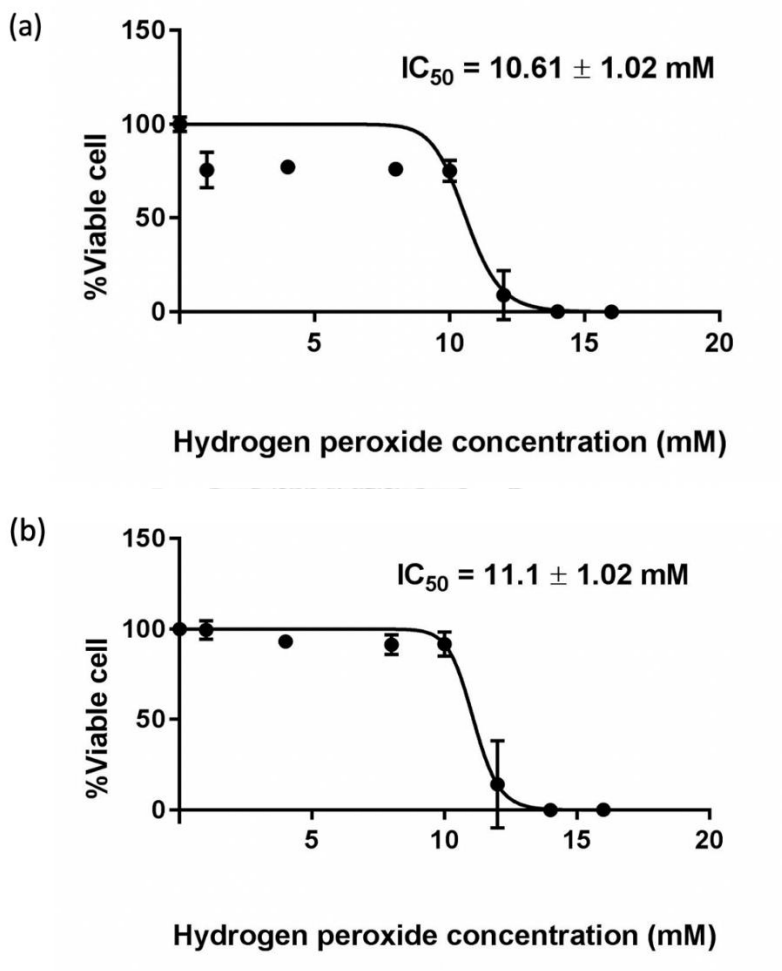


**Figure 10** Determination of $IC_{50}$ for *E. coli* BL21 carrying empty pET15b vector upon oxidative stress induced by $H_2O_2$ at (a) 24 hours, and (b) 42 hours.

**4.5.3 Oxidative stress**

Controls and four expressing cells were cultured under oxidative stress, induced by $H_2O_2$ (a concentration corresponds to $IC_{50}$) for 48 hours. Two experimental sets were performed with or without IPTG. The growth of all kind of cells were observed. Without IPTG, all kind of cells were similar. In contrary, in the presence of IPTG, growth rate was somehow different (figure 11a). GST_0729 and GST_3557 had the higher growth ability under oxidative stress, followed by GST_1478, GST_0647 and pGEX6P-1, respectively. The empty vector control, pET15b, had the lowest growth rate under oxidative stress (figure 11b). Based on $OD_{600}$ value, it should be mentioned that the overall growth profile of all recombinants upon IPTG adding was lower than without IPTG.

After stress treatment for 48 hours, the cells were further examined survival rate. In a set of no IPTG adding, amongst six transformants, all *Halothece* GSTs expressing cells had higher survival rate (figure 12a). Likewise, all *Halothece* GSTs expressing cells had higher survival rate with IPTG adding. These results suggested that all *Halothece* GSTs are capable of supporting the viable ability of the cell against oxidative stress induced by $H_2O_2$. Amongst four *Halothece* GST expressing cells, GST_3557 performed the highest survival cells. The viable cell count of GST_3557 was greater than pET15b, approximately 12 folds (figure 12b).

Oxidative stress affects microbial cell growth, cellular metabolism or even lead to cell death. It causes by the imbalance of ROS and cellular antioxidant (Imlay, 2019). Excess generation of ROS disrupts cellular redox homeostasis, increases the accumulation of misfolded or unfolded proteins and causes the defective reaction. These adverse effects resulted in the damages of cellular components (Birben et al., 2012). A number of antioxidants and detoxification enzymes are in responsible to combat the oxidative stress. GST is one of cellular detoxification enzyme in respond to oxidative stress. Glutathione acts as ROS scavenger by conjugation reaction catalyzed by GST. Thus, ROS are changed into stable form to prevent defective reaction (Zhang et al., 2018). The result obtained here supported the hypothesis that GST from extremophile *Halothece* sp. PCC7418 had vital function in a heterologous expression system. Specifically, GST_3557 contributes as the best amongst four

*Halothece* GSTs. These results also implicated that GST_3557 should be the most crucial isozyme responses against oxidative stress.

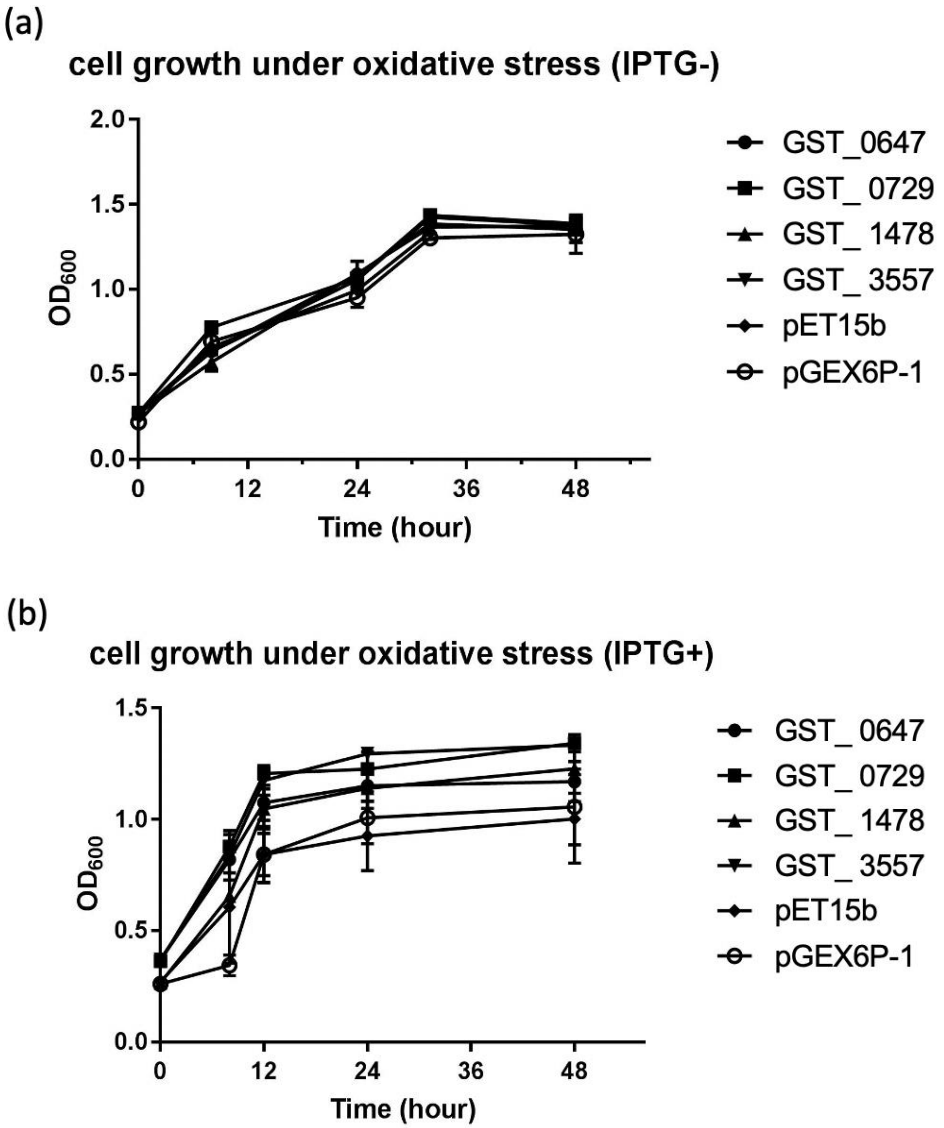


**Figure 11** Growth profile of recombinant *E. coli* BL21 under oxidative stress-induced by $H_2O_2$. A concentration of IC50 was used for 48 hours: (a) without IPTG, and (b) with 0.5 mM IPTG. The growth was measure via $OD_{600}$.

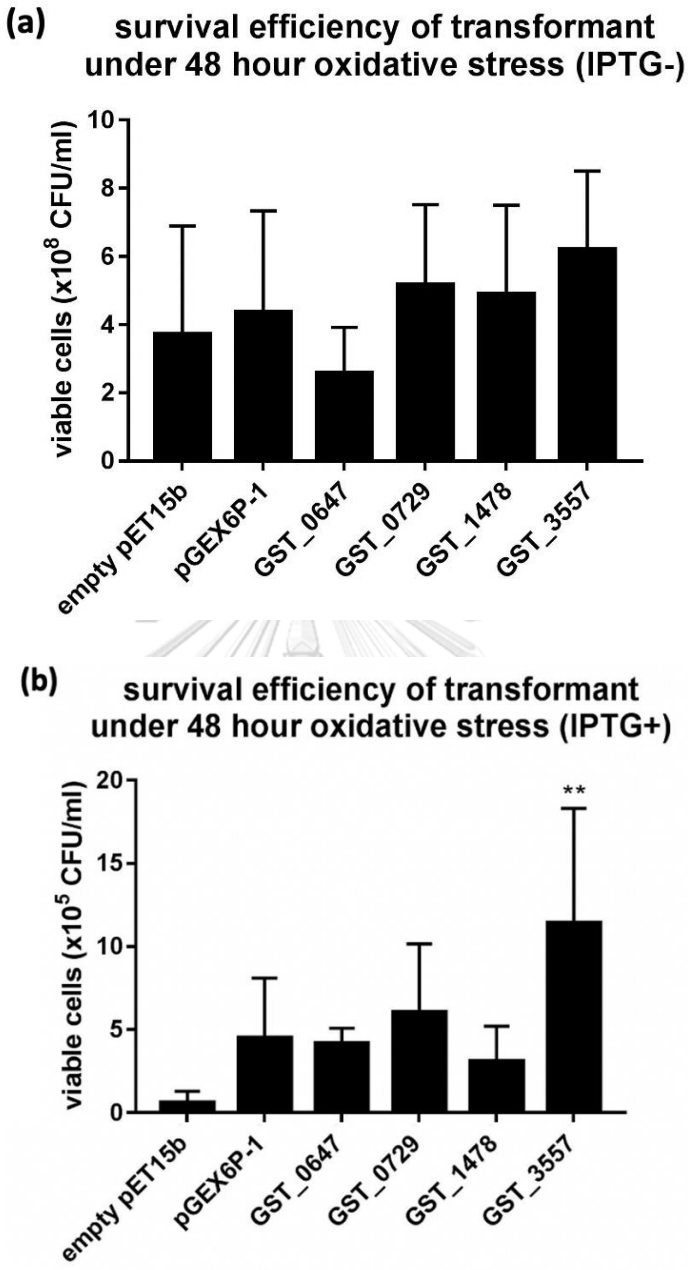


**Figure 12** Survival efficiency of expressing cells containing each recombinant GST gene after treated under oxidative stress for 48 hours, (a) no IPTG added, and (b) added 0.5mM IPTG. The stars shown significant level, * is sig < 0.05 and ** is sig < 0.01, at 95% confidence level.

**4.5.4 Metal stress**

After testing the survival efficiency of each *Halothece* GSTs under salt and oxidative stresses (Figure 9 and 12), GST_3557 was selected as a best candidate because of the highest survival efficiency against stress treatments. Thus, the *E. coli* BL21 expressing GST_3557 was used for metal stress tolerances. This experiment performed as similar way with section3.11.1 (with or without IPTG). The expressing cells containing empty pET15b and pGEX6P-1 vector were also tested. The wild-type *E. coli* ATCC8739 was used as negative control. Tested materials were copper and silver discs, and a paper disc served as a negative control.

The result shown that *E. coli* BL21 expressing GST_3557 survived better than wild-type *E. coli*, according to smaller inhibition zone surrounding copper and silver discs. Without IPTG, *E. coli* BL21 expressing GST_3557 resisted to both tested metals better than *E. coli* carrying empty pET15b and pGEX6P-1 vectors. In another case, with 0.5 mM IPTG, *E. coli* expressing GST_3557 resisted to silver better than other *E. coli* clone but resisted to copper not different from *E. coli* carrying empty pET15b and pGEX6P-1 vector. All tested *E. coli* cells grew surround pound paper disc with 0 mm inhibition zone (Figure 13).

Silver and copper are the representative metals that could be toxic to the cells. High concentration of copper and silver enhance lipid oxidation, trigger the generation of ROS and lead to oxidative stress; finally, resulted in cell mortality (Adeyemi et al., 2020; Saporito-Magriñá et al., 2018). There are various cellular mechanisms response against the toxicity and oxidative stress induced by metals, including enzyme SOD and other antioxidants such as GSH. GST is one of well-acceptable phase II detoxification enzyme. This enzyme prevents the metal toxicity by catalyzing conjugation of metal ion and GSH. In fact, GST plays a detoxification role not against only silver and copper, but also other heavy metals, such as cadmium, nickel and aluminum (Hamed et al., 2019; Jan et al., 2019; Singh et al., 2018). According to this study, the result confirms that GSTs play a vital role in cell survival, not only against hydroperoxide and ROS, but also against the toxic metal ions, such as copper and silver.

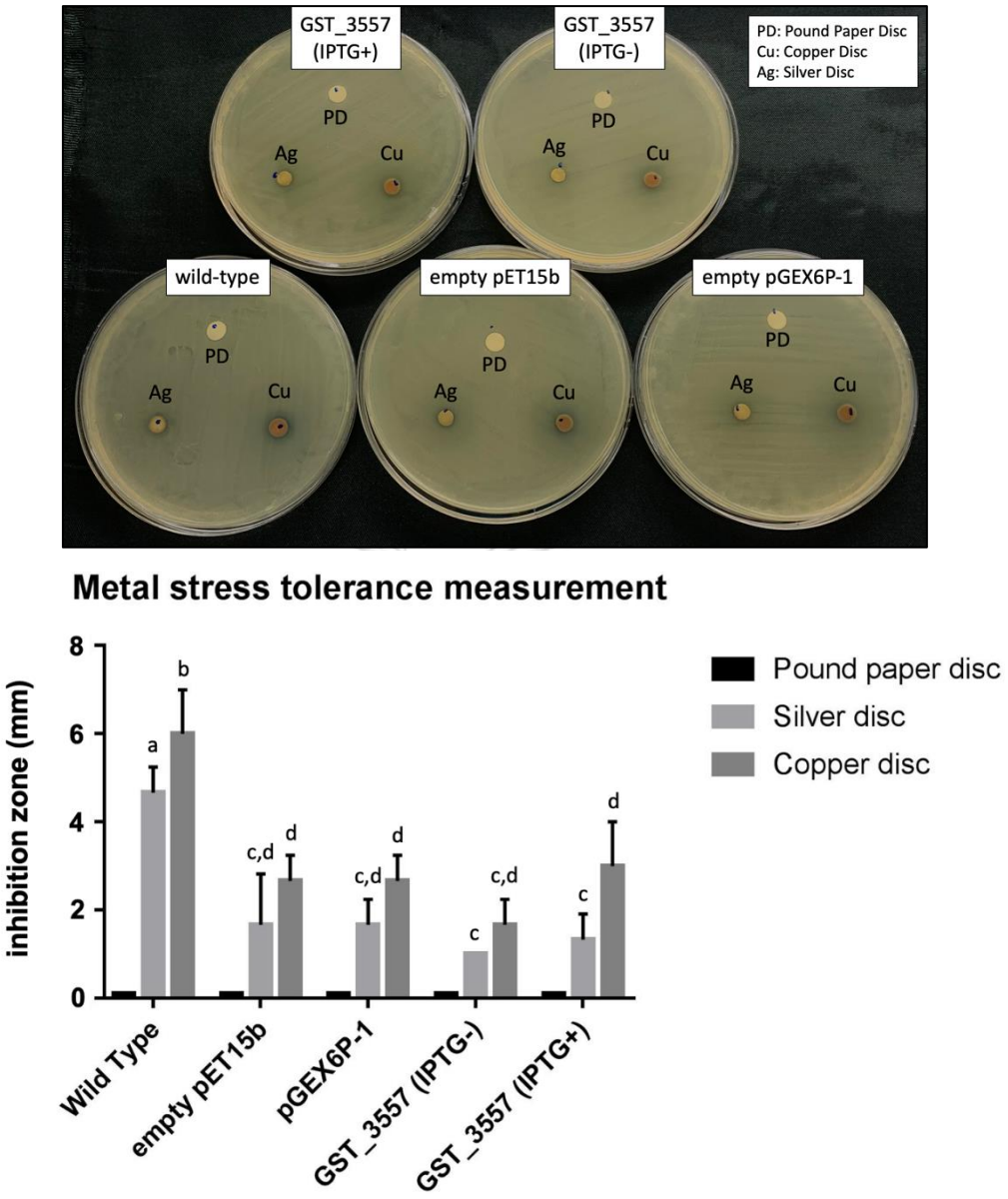


**Figure 13** Inhibition zone of the expressing cells GST_3557 (without IPTG and 0.5 mM IPTG added), empty pET151b and empty pGEX6P-1 vectors, and wild-type *E. coli* ATCC8739 against silver and copper induced stress. A Pound paper disc was used as a negative control. Different letters indicate the significant differences ($p<0.05$).

**4.6 Purification of *Halothece* GSTs**

Based on *in vivo* stress tolerance in a heterologous expression system, the cell expressing GST_3557 confers the best performance under all stress tested (Figure9, 12 and 13). Thus, GST_3557 was selected for further functional characterization. In addition, GST_0729, which also supported a vital function in a heterologous expression system, was also used for functional characterization. Expression of these two proteins were performed by adding IPTG (at a final concentration of 0.5 mM). Thereafter, both expressing cells were cultured at 30°C, for 6 hours. The cells were harvested and extracted total protein by sonication. Supernatant solutions were preserved at -20°C before used in the next step.

Crude protein GST_3557 (7 mg) and GST_0729 (10 mg) were purified using His-trap™ affinity chromatography column (GE Healthcare, USA). Tris-Cl buffer (100 mM) containing 100 mM NaCl and 40 mM imidazole was used as binding/washing buffer. Two *Halothece* GST fusion proteins were eluted from the column using 100 mM tris-Cl buffer containing imidazole (200, 300 and 500 mM for first, second and third elusion, respectively). The flow-through, washed and eluted fraction were separately collected and analyzed for protein purity by SDS-PAGE. The result shown that GST_0729- and GST_3557-fusion proteins were purified by affinity chromatography (Figure 14). Thereafter, the three eluted fractions of each protein were pooled together; then, desalted by dialysis at 4°C, using cellophane membrane with 3,500 Da cutoff. Lastly, the purified GST_0729 or GST_3557 was analyzed to confirm the purity by SDS-PAGE and Western blot analysis, respectively.

The SDS-PAGE analysis revealed that purified GST_3557 comprised a single band. The imidazole and other small-molecule contaminated proteins were removed from the protein sample and ready for performing enzymatic activity assays (Figure 15a). However, the GST_0729 was precipitated during dialysis (Figure 15b). One of the plausible reasons to explain this phenomenon is this protein might change its conformation when salt was removed (Table 6). Western blot analysis, using anti 6-His-tag as primary antibody and anti-mouse IgG conjugated with HRP as secondary antibody was performed. According to the precipitation of GST_0729

during dialysis, the pre-dialysis purified protein was used instead. The specific signals of purified GST_0729 and GST_3557 were detected on PVDF membrane (Figure 15).

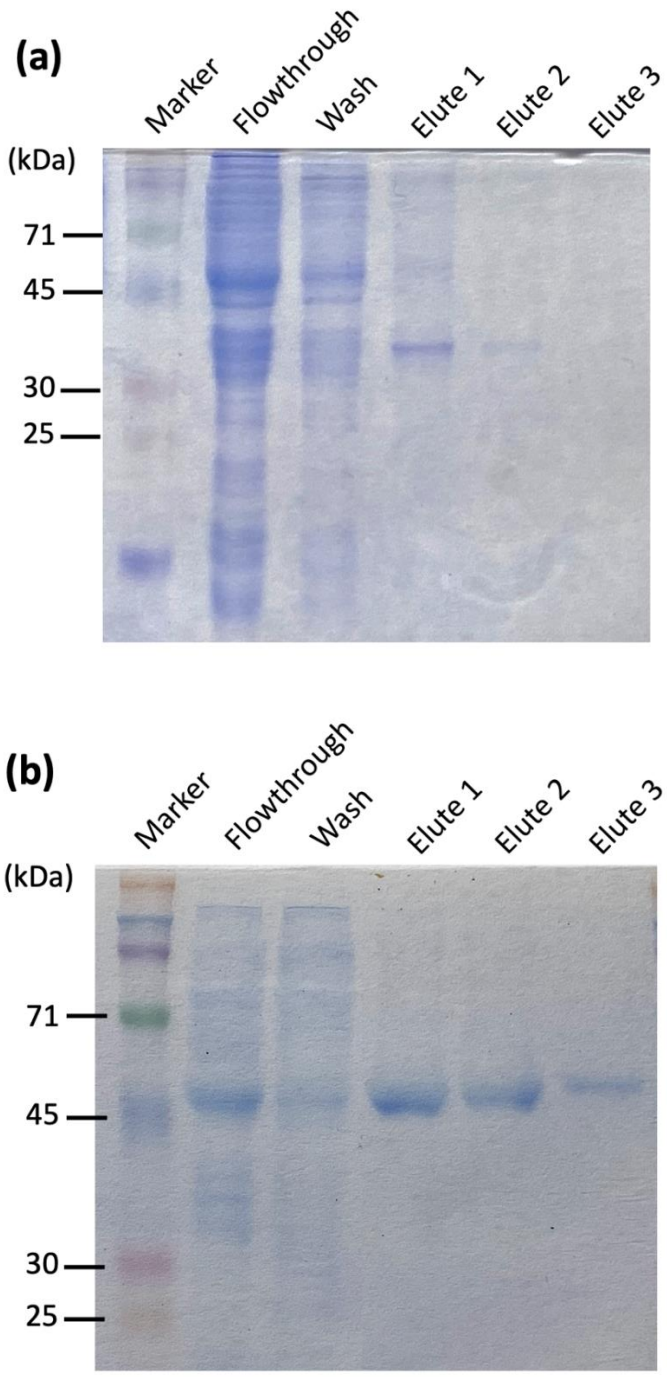


**Figure 14** SDS-PAGE analysis of (a) GST_3557 and (b) GST_0729 from batch purification using His-trap affinity chromatography. Elute 1, Elute 2 and Elute 3 were the purified protein fractions that eluted using 200, 300 and 500mM imidazole, respectively.

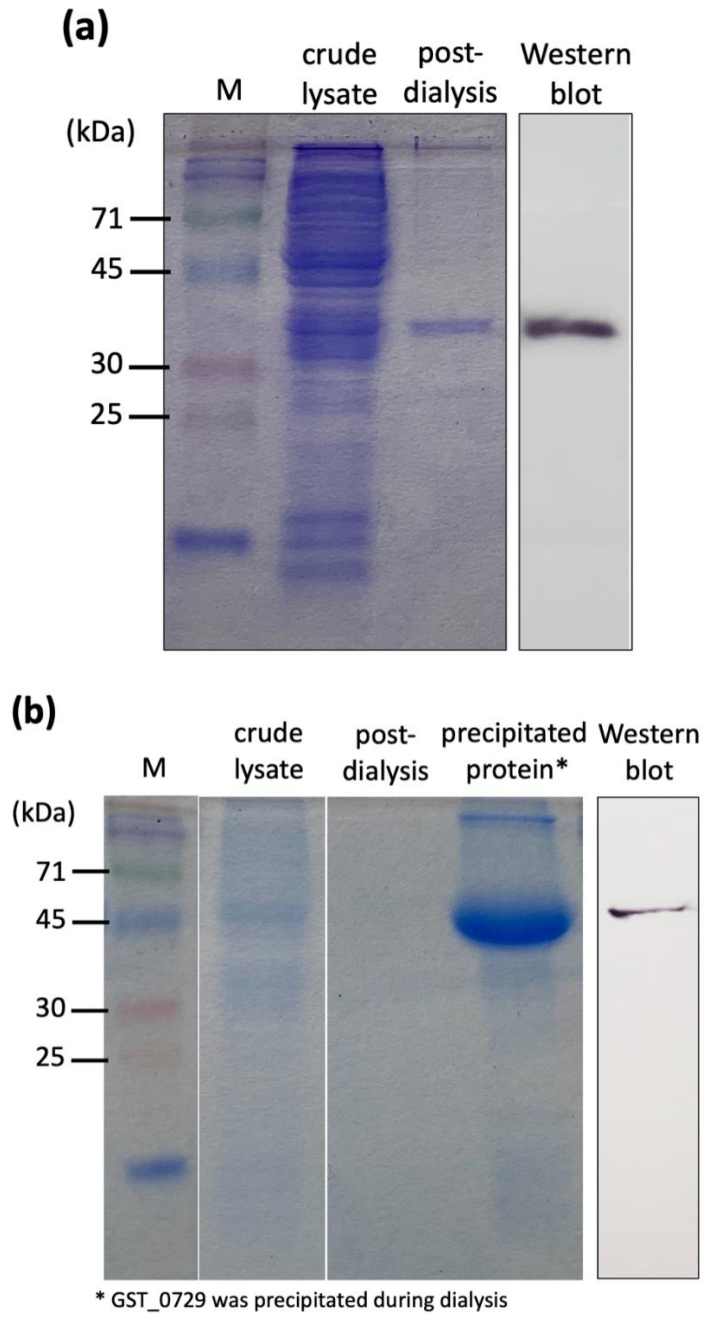


**Figure 15** SDS-PAGE and Western blot analysis of purified proteins (a) GST_3557 and (b) GST_0729. For Western blotting, the anti-6-His tag and anti-mouse HRP conjugated were used as a primary and secondary antibody, respectively. The proteins were blotted on PVDF membrane and visualized by HRP-conjugated color reagent (4-chloro-1-naphtahol and $H_2O_2$).

## 4.7 Functional characterization

### 4.7.1 GST activity

This enzymatic assay was performed using the purified recombinant GST_3557. The reactions were performed using a series of three buffers with a pH range of 5.5-10.5 to determine pH dependency of enzyme activity. Activity of GST_3557 preferred a mild alkali condition (Figure16). The sodium-phosphates buffer at pH 7.5 was the optimal buffer and pH condition. GST displayed the highest activity compared to other buffers and pH (Figure 16). In comparison with other GSTs, the suitable buffer for GST activity assay was either sodium-phosphate or potassium-phosphate buffer. The optimal pH for GST activity can be in a range of 6.5 to 7.5 (Table 7). The optimal pH for *Halothece* GST_3557 is similar to halophilic-psychrophilic bacterium *Halomonas* sp. ANT108 and plant-pathogenic fungus *Alternaria brassicicola* (Calmes et al., 2015; Hou et al., 2019). While the optimal pH of GSTs in cyanobacteria and microalgae was reported around 6.5, except in case of *Synechocystis* sp. PCC 6803 GSTs (sll0067 and sll1545). Both GSTs were performed the best activity at pH 8.0 (Pandey et al., 2015a; Pandey et al., 2015b). In addition, GST_3557 also performed high activity in Tris-Cl buffer pH 8.4. Thus, both pH 7.5 (sodium-phosphate) and pH 8.4 (Tris-Cl) were further determined kinetics parameters.

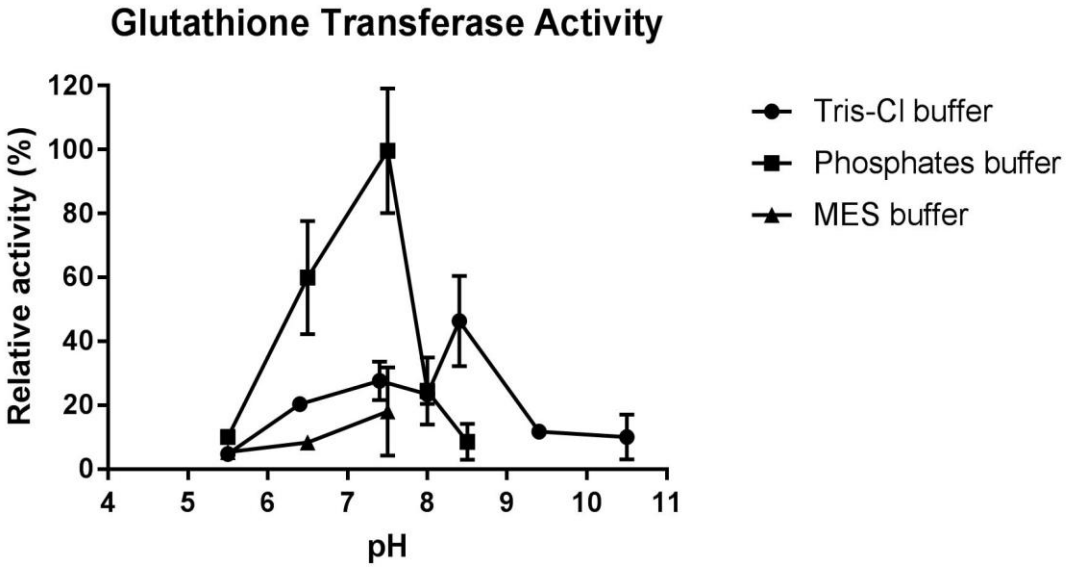


**Figure 16** Effect of pH on GST activity of GST_3557. The purified GST_3557 was used and GSH and CDNB were served as substrates. The assay was performed under room temperature (25°C). The conjugated product was measure via $OD_{340}$.

**Table 7** Comparison of optimal pH and buffer for GST activity assay, using CDNB and GSH as substrates, in eight representative members of bacteria, cyanobacteria, microalgae, fungi, plants and animals.

| organisms | pH | buffer | references |
|---|---|---|---|
| *Synechocystis* sp. PCC 6803 (sll0067) (cyanobacteria) | 8.0 | 0.05M potassium-phosphate | (Pandey et al., 2015b) |
| *Synechocystis* sp. PCC 6803 (sll1545) (cyanobacteria) | 8.0 | 0.1M sodium-phosphate | (Pandey et al., 2015a) |
| *Synechococcus elongatus* PCC6301 (SeGST) (cyanobacteria) | 6.5 | 0.1M potassium-phosphate | (Wiktelius & Stenberg, 2007) |
| *Chlamydomonas reinhardtii* (CrGST10) (green microalgae) | 6.5 | 0.1M potassium-phosphate | (Chatzikonstantinou et al., 2017) |
| *Chlamydomonas reinhardtii* (CrGST7) (green microalgae) | 6.5 | 0.1M potassium-phosphate | (Chatzikonstantinou et al., 2017) |
| *Halomonas* sp. ANT108 (rHsGST) (bacteria) | 7.5 | 0.1M sodium-phosphate | (Hou et al., 2019) |
| *Agrobacterium tumefaciens* (AtuGSTH1-1) (bacteria) | 6.5 | 0.1M potassium-phosphate | (Skopelitou et al., 2012) |
| *Alternaria brassicicola* (AbGTT1.2) (fungi) | 7.5 | 0.1M phosphate | (Calmes et al., 2015) |
| *Arabidopsis thaliana* (higher plant) | 7.0 | 0.2M potassium-phosphate | (Bartling et al., 1993) |
| *Schistosoma japonicum* (Sj26GST) (flat worm) | 7.4 | sodium-phosphates | (Habig et al., 1974) |
| ***Halothece* sp. PCC7418 (GST_3557) (cyanobacteria)** | **7.5** | **0.1M sodium-phosphate** | **this study** |

In order to examine the steady-state kinetic parameters of the *Halothece* GSTs, the apparent $K_m$ values for GSH and CDNB were separately determined by varying the concentration in one of them and keeping constant the other. Kinetic parameters were calculated from Michaelis-Menten equation from GraphPad Prism 7 (http://www.grapgpad.com/scientific-software/prism/).

At pH 7.5 (sodium-phosphate buffer), $K_m$ and $V_{max}$ for GSH were 0.74±0.29 and 0.13±0.03, respectively (Figure 17a). The $K_m$ and $V_{max}$ for CDNB were 0.14±0.02 and 0.16±0.07, respectively (Figure 17b). The kinetics parameters of GST_3557 was compared to GST from other organisms (Table 8). The $K_m$ of GST_3557 for GSH is similar to GST from cyanobacterium *Synechococcus elongatus* PCC6301 (approximately 0.75 mM) (Wiktelius & Stenberg, 2007). In addition, the $K_m$ of GST_3557 for GSH was lower than GST from cyanobacterium *Synechocystis* sp. PCC 6803 (sll0067 and sll1545) and plan-pathogenic fungi *Alternaria brassicicola* (AbGTT1.2). However, the $V_{max}$ for GST_3557 for GSH was lower than GST from cyanobacterium *Synechocystis* sp. PCC 6803 (sll0067 and sll1545) and halophilic-psychrophilic bacteria *Halomonas* sp. ANT108 (rHsGST). The $K_m$ of GST_3557 for CDNB was the lowest among compared organisms (Table 9). The result suggested that GST_3557 had very high affinity for electrophilic substrate CDNB. In contrary, the $V_{max}$ of GST_3557 for CDNB is lower than other compared organisms.

In another condition, at pH 8.4 (Tris-Cl buffer), $K_m$ and $V_{max}$ of GST_3557 for CDNB were 0.19±0.13 and 0.69±0.17, respectively, while the $K_m$ and $V_{max}$ for GSH were 1.54±4.11 and 1.62±3.33, respectively (Table 10). $V_{max}$ for both GSH and CDNB at pH 8.4 was higher than at pH 7.5; while the $K_m$ for both GSH and CDNB at pH 8.4 was slightly higher than at pH 7.5. Therefore, at more alkali condition, GST_3557 likely binds to CDNB and catalyzes the reaction faster, but the affinity is lower.

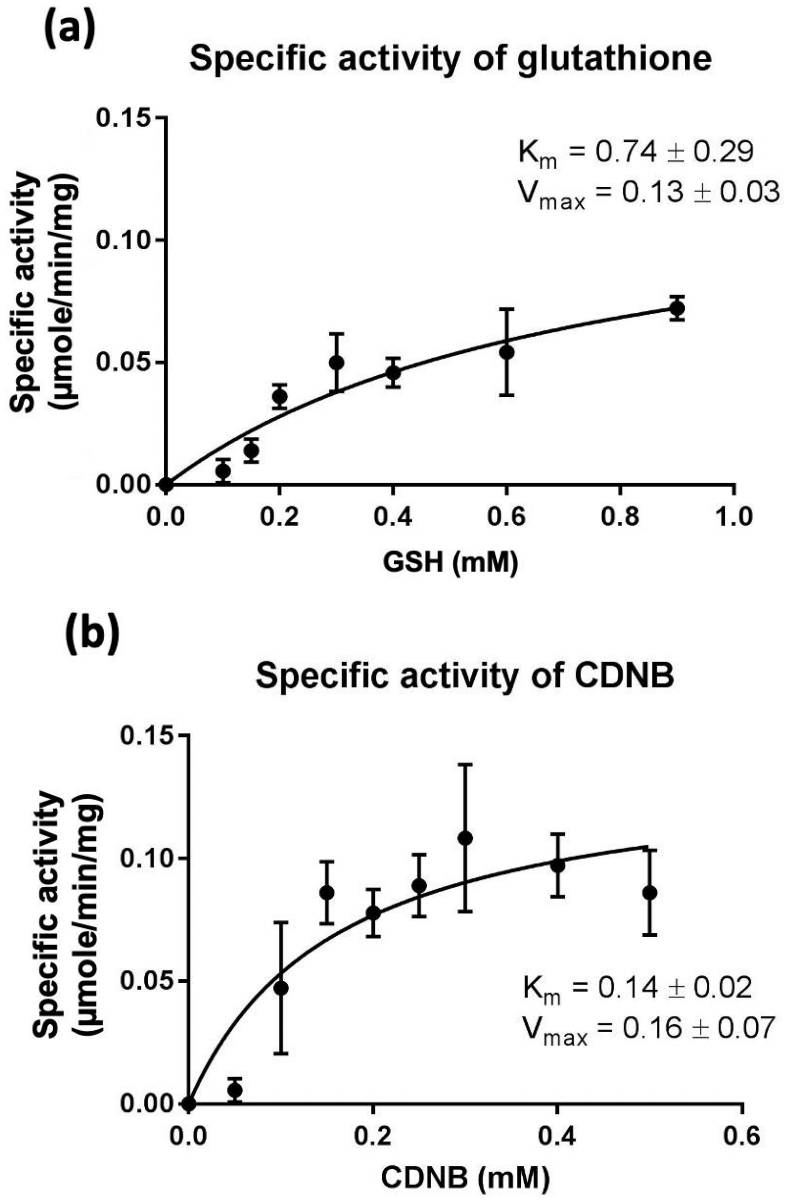


**Figure 17** Kinetics parameters of the recombinant GST_3557 under sodium-phosphate buffer pH 7.5 for (a) GSH and (b) CDNB. The kinetics parameters were analyzed from the Michaelis-Menten kinetics plot using Graph-pad Prism 7.0 software. All assays were performed in three replicates.

**Table 8** Kinetic parameters for GSH from eight representative members of bacteria, cyanobacteria, microalgae, fungi, plants and animals.

| Original organisms | $K_m$ (mM) | $V_{max}$ (μmol/min/mg) | references |
|---|---|---|---|
| *Synechocystis* sp. PCC 6803 (sll0067) (cyanobacteria) | 0.92 | 12.92 | (Pandey et al., 2015b) |
| *Synechocystis* sp. PCC 6803 (sll1545) (cyanobacteria) | 1.50 | 23.22 | (Pandey et al., 2015a) |
| *Synechococcus elongatus* PCC6301 (SeGST) (cyanobacteria) | 0.75 | NR | (Wiktelius & Stenberg, 2007) |
| *Chlamydomonas reinhardtii* (CrGST10) (green microalgae) | 0.32 | NR | (Chatzikonstantinou et al., 2017) |
| *Chlamydomonas reinhardtii* (CrGST7) (green microalgae) | 0.31 | NR | (Chatzikonstantinou et al., 2017) |
| *Halomonas* sp. ANT108 (rHsGST) (bacteria) | 0.27 | 0.24 | (Hou et al., 2019) |
| *Agrobacterium tumefaciens* (AtuGSTH1-1) (bacteria) | 0.30 | NR | (Skopelitou et al., 2012) |
| *Alternaria brassicicola* (AbGTT1.2) (fungi) | 1.33 | NR | (Calmes et al., 2015) |
| *Arabidopsis thaliana* (higher plant) | 0.08 | NR | (Bartling et al., 1993) |
| *Schistosoma japonicum* (Sj26GST) (flat worm) | 0.43 | NR | (Walker et al., 1993) |
| ***Halothece* sp. PCC7418 (GST_3557) (cyanobacteria)** | **0.74** | **0.13** | **this study** |

**NR**: No Report

**Table 9** Kinetic parameters for GSH from seven representative members of bacteria, cyanobacteria, microalgae, fungi, plants and animals.

| Original organisms | $K_m$ (mM) | $V_{max}$ (μmol/min/mg) | references |
|---|---|---|---|
| *Synechocystis* sp. PCC 6803 (sll0067) (cyanobacteria) | NR | 4.62 | (Pandey et al., 2015b) |
| *Chlamydomonas reinhardtii* (CrGST10) (green microalgae) | 1.41 | 5.5 | (Chatzikonstantinou et al., 2017) |
| *Chlamydomonas reinhardtii* (CrGST7) (green microalgae) | 1.00 | 13.4 | (Chatzikonstantinou et al., 2017) |
| *Halomonas* sp. ANT108 (rHsGST) (bacteria) | 2.86 | 0.71 | (Hou et al., 2019) |
| *Agrobacterium tumefaciens* (AtuGSTH1-1) (bacteria) | 1.50 | NR | (Skopelitou et al., 2012) |
| *Alternaria brassicicola* (AbGTT1.2) (fungi) | 1.82 | NR | (Calmes et al., 2015) |
| *Arabidopsis thaliana* (higher plant) | 10.0 | NR | (Bartling et al., 1993) |
| *Schistosoma japonicum* (Sj26GST) (flat worm) | 2.68 | NR | (Walker et al., 1993) |
| ***Halothece* sp. PCC7418 (GST_3557) (cyanobacteria)** | **0.14** | **0.16** | **this study** |

**NR**: No Report

**Table 10** Kinetic parameters of GST_3557 at pH 7.5 and 8.4

| Buffer and pH condition | substrate | $K_m$ (mM) | $V_{max}$ (μmol/min/mg) |
|---|---|---|---|
| sodium phosphate, pH 7.5 | GSH | 0.74±0.29 | 0.13±0.03 |
| sodium phosphate, pH 7.5 | CDNB | 0.14±0.02 | 0.16±0.07 |
| Tris-Cl, pH 8.4 | GSH | 1.54±4.11* | 1.62±3.33* |
| Tris-Cl, pH 8.4 | CDNB | 0.19±0.13 | 0.69±0.17 |

* data from two replicates

GST is a set of multifunctional enzymes. Basically, enzyme activity was performed using CDNB as a universal substrate because most GSTs utilizes CDNB as substrate to conjugate with GST (Hou et al., 2019). Conjugation between GSH and electrophilic substrates, including xenobiotics, hydro-peroxides and other toxic compounds were also widely reported (Perperopoulou et al., 2018; Zhang et al., 2018). Conjugation between GST and toxic substrates is an important function in phase II cellular detoxification system against oxidative stress. The toxic electrophilic substrates and ROS are finally detoxified and prevent the defective reactions to the cellular components (Hamed et al., 2019). There are various substrates of GST, such as $H_2O_2$, 1-fluoro-2,4-dinitrobenzene (FDNB), ethacrynic acid, cumene hydroperoxide (CuOOH) and Bromosulftalein 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB). For the kinetic parameters observed in this study, $V_{max}$ is the maximum rate of reaction, when the enzyme is saturated with substrate. The higher $V_{max}$ value reflects efficiency of enzyme to catalyze the reaction faster. Another kinetics parameter observed in this study is $K_m$, which is the concentration of substrate permitting the enzyme to achieve a half of $V_{max}$. The lower value of $K_m$ refers to affinity of enzyme binding to substrate better. From this study, both $K_m$ and $V_{max}$ for CDNB were higher than GSH. This can be implied that GST_3557 prefers the binding with CDNB better than GSH. Moreover, this GST performed the best GST activity at a mild alkali condition, but the activity was declined when pH is higher than 8.4 (Figure 16).

**4.7.2 Effect of salt on GST activity**

This activity assay was performed in optimal buffer in the presence of NaCl (up to 2 M). The result revealed that GST_3557 still performed high efficiency even under the presence of NaCl up to 2M. The activity retained approximately 60% under the presence of 2 M NaCl (Figure 18). High salt concentration affects the stability and/or conformation of enzyme resulted in inhibition of enzyme activity (Lanyi & Stevenson, 1969). GSTs displayed the declined activity in the presence of salts (Stevens et al., 2000). In contrary, GST_3557 lost its activity only about 40%. Thus, GST_3556 can function under high salt condition. GST_3557 possibly contains special characteristics in its structure, resulting a robust function under salt-stress condition.

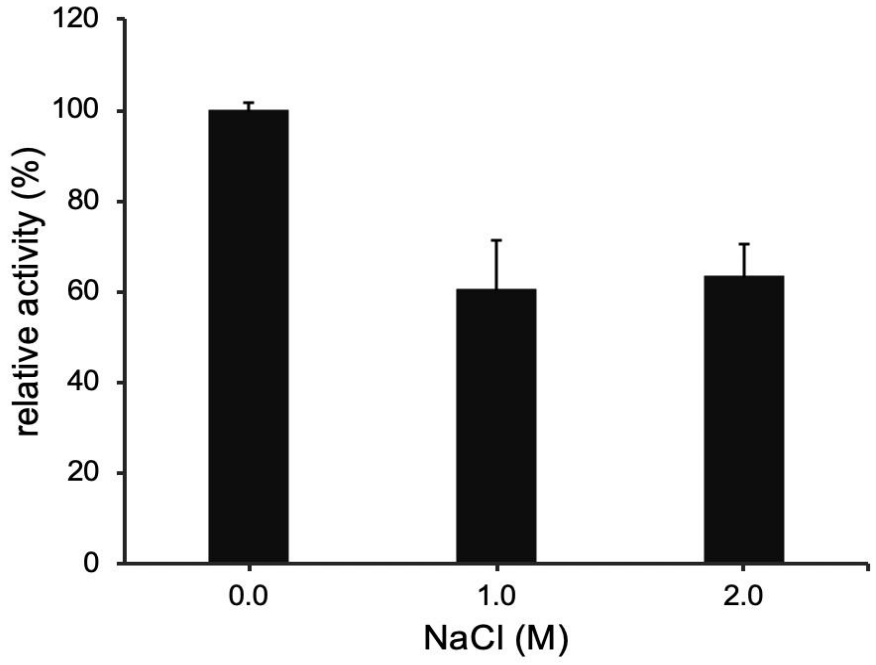


**Figure 18** Effect of salt on GST activity of GST_3557. The enzyme assay was performed in sodium phosphate buffer pH 7.5, using GSH and CDNB as substrates, under room temperature (25°C). The conjugated product was measure via $OD_{340}$.

**4.7.3 Peroxidase activity**

The assay was performed in two set of buffers, which were sodium-phosphate (pH 5.5-8.5) and Tris-Cl (pH6.4-9.4). No color changes during 5 minutes of determination in all reactions tested (Figure 19). This can be implied that GST_3557 does not exhibit peroxidase activity.

In other organisms, there are a number of studies suggest that GST in certain organisms exhibits peroxidase activity. This activity likely use to scavenge and/or degrade the hydro-peroxide substrates, such as $H_2O_2$ and CuOOH (Hossain et al., 2015; Pandey et al., 2017a; Theoharaki et al., 2019). GST_3557 expressed against the present of $H_2O_2$ but not contain the ability to scavenge $H_2O_2$ directly. This isozyme might be function in the conjugation of other toxic substrates generated under the oxidative stress, induced by $H_2O_2$, and prevent the defective reactions to cellular protein components by other mechanisms.

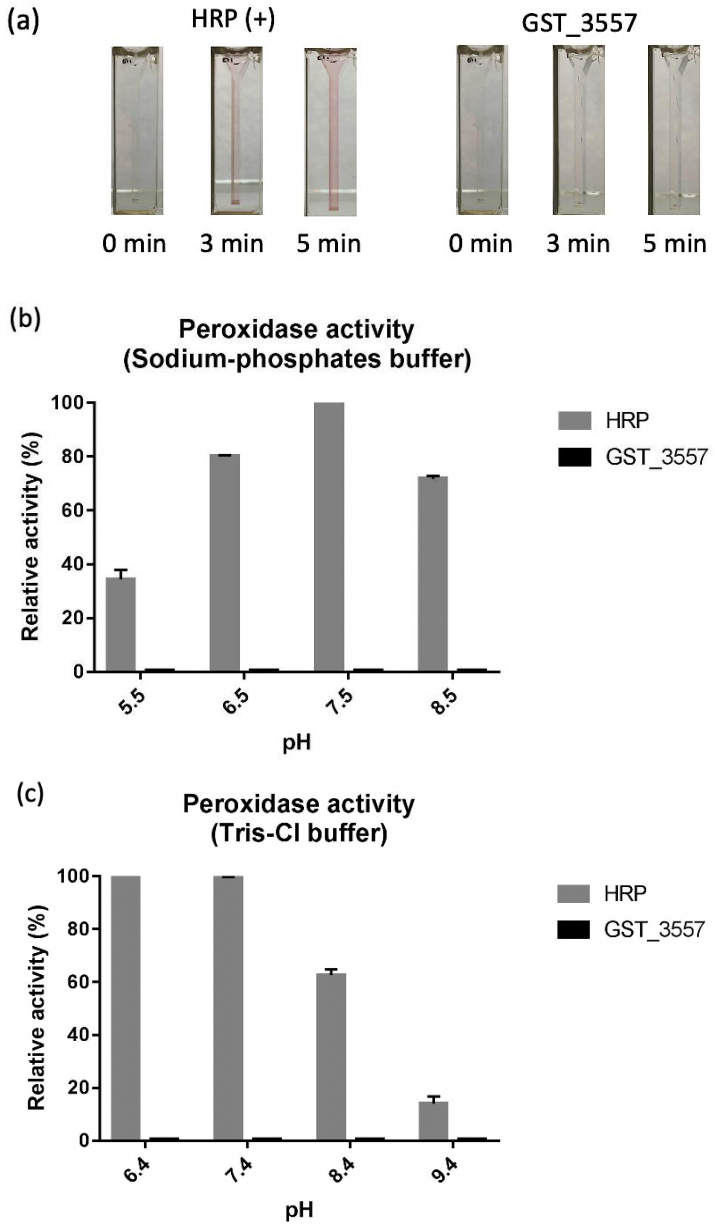


**Figure 19** Determination of peroxidase activity of GST_3557: (a) The color changing of peroxidase assay reaction, in sodium-phosphates buffer pH 7.5, after adding HRP or GST_3557 during 0-5 minutes of determination. The relative activity was compared between HRP (positive control) and GST_3557 in (b) sodium-phosphates buffer, pH 5.5-8.5 and (c) tris-Cl buffer pH 6.4-9.4.

# CHAPTER V
# CONCLUSIONS

(I) In *Halothece* genome, it comprised of at least four GSTs with different physicochemical properties and domain architectures. GST_0729 and GST_3557 consisted of low complexity region with some special features instead of the C domain.

(II) Phylogenetic analysis revealed that four *Halothece* GSTs were diverse in different clades. GST_0647 and GST_3557 were closet to Chi-class and Zeta-class GSTs, respectively. The other two *Halothece* GSTs cannot be defined the class designations to any GSTs classified to date.

(III) *Halothece* GSTs play a vital function in a heterologous system. The expressing cells carrying GST_3557 significantly survived under oxidative stress-induced by $H_2O_2$, approximately 12 folds, compared with the empty vector control.

(IV) *Halothece* GSTs also play a vital role under salt stress. Likewise, the expressing cells carrying GST_3557 performed the best performance amongst other.



(V) GST_3557 preferred a mild alkali condition. Kinetic measurements revealed that GST_3557 had high affinity for electrophilic substrate.

(VI) GST_3557 lack of peroxidase activity. The activity assay using $H_2O_2$ as substrate is resulted in negative.

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**Thesis Reference**

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# APPENDICES

## 1. Nucleotide sequence and primer design

**1.1 PCC7418_0647** (552 nucleotides)

ATGCTTAAACTATATGGTGCAACCAGAAGTCGCGCCGCGATCGCGCGATGGTATTTAGAAGAAC
TGAAAGTTCCCTACGAATTTGTTGAACTGGATATGGCAAATGGGGAACATCGCAAACCACCATT
TCTTGCTATTAACCCCATGGGAAAAGTTCCCGCGATTGAAGATAATGGCTTTTCTTTATGGGAG
TCGGGAGCAATTCTTTTATATTTAGCCGATCACTACGAACCCGAACCACTAACTCCACAAAAAC
GGGCAATTCTGAATCAATGGATTTTATTTGCGAATTCAACCCTTAGCATTGGTATTTTTATCGAG
AGTAACCGCGATAATGAAATGCCAAAACTCTTTCCCCCCTTAAACGATCATTTAACCCAACACG
ACTACTTAGTTGATGATCAATTTAGTGCTGCTGATGTTGCTGTCGGGGCTTATTTAGCTTATATG
CCCAGAATGTTACAACTGGATTTTTCCGACTATCCTGCTATTGCTAAATATGTGGAAAATCTCTC
CCAACGTCCTGCATTTAAAACAGGAATGGGCTTCTAA

| primer | sequence | length (bp) | Tm (°C) |
|---|---|---|---|
| PCR_GST0647-Forward | 5’ – GCGATTGAAGATAATGGCT– 3’ | 19 | 53.0 |
| PCR_GST0647-Reverse | 5’ – ACATTCTGGGCATATAAGCT– 3’ | 20 | 54.3 |



**1.2 PCC7418_0729** (1,200 nucleotides)

ATGCAGGCACTGAGTTGGGAAGAATTAGAAAACCGTACAAATTTTGAAATTGATCGCGTTAATG
GACCGACGAATGCACAATCTCGTTTACGCTTATTTGGGCGCGATGAATCGGAGGTTCGAGTGAC
GTTATACCGTGACCATCATGCTTGGTGTCCCTATTGTCAGAAAGTTTGGTTATGGTTAGAAGAA
AAACAAGTTCCCTATCGTGTGGAAAAAGTCACGATGTTTTGCTATGGGGATAAAGAGCGTTGGT
ATAAGCAGATTGTTCCTTCAGGGATGTTACCTGCGTTAAAACTCGATGATCGTTTGCTTACTGA
AAGTGATGATATTTTAAGCCAACTTGAGCAAACCTTCGGAACGCTGGGTTATAGTATGAACGAT
CGCGCCAGTATTGCCCTACGGAAGTTAGAACGACTGTTATTTCGGGCGTGGTGTAGTTGGTTAT
GTGTTCCTGCGCGATCGCGCCGTGAAGACCAGTATAACCGCCAACAGTTTACGGATGTGGTCTC
CCAAGTTGAGGACGCGCTACAACAAACCCCGGGTCCTTATTTCCGAGACAGCTTTAGCATTATT

GATCTTATCTTTACCCCGTTTCTGGAACGGATGAACGCCAGTTTATTCTATTACAAAGGGTACTC
CCTACGAGAAGAAAACCCTCAACTGGGCTTATGGTTTGATGGGATGGAACAGCGATCCACCTAT
CGCGGAACGCAAAGTGATTTTCATACCCACGTTCATGATTTACCCCCACAGATGGGCGGTTGCT
ATGCTAACGATGAACCGCAAACAAAACTGAATCAAGCACGGGTGGATCAGGGACCTTGGCTAGG
TTTACCTGATGTGATGTATCCTGAACCCGAAACCTCGCGAGAAGAAGCCTTACAACGGGTTTTA
AAGCATCGAGAGAACTTGTTAAACGTGAATCCAGCCTCAGAAGACTTATTTGAGGAAGCCTTGC
GCTGTGCGTTAACCAATTTGATTACTGGTGAAGTGTGTTCTCCCCCTGCTGGATCAGCATCTGC
ATTAAGATATTTGCGCGATCGCGTGAGTGTTCCTAGAGATATGTCGATTTATGCAGCCAAACGC
TTACGAGAAGCCTTAGAAAACACCGCCAGCTTAGCTGGAGACGAACAAGGAACACCGATTCCAG
TTCGACATCGGCGCGATCAAGATCCCGCGAATTTTGCAAAAGTTTGA

| primer | sequence | length (bp) | Tm (°C) |
|---|---|---|---|
| PCR_GST0729-Forward | 5’ – GTCCTTATTTCCGAGACAGC– 3’ | 20 | 58.4 |
| PCR_GST0729-Reverse | 5’ – ACATCAGGTAAACCTAGCCA– 3’ | 20 | 56.4 |

**1.3 PCC7418_1478** (561 nucleotides)

ATGAAACTTTATTATCTTCCGTTAACCCGAGCCAGTCGCCCTCATTGGCTATTAGAAGAACTGG
AAATTTCCTATGAATTAATTCAAGTGACCCCTGATGAAATGTCGGAGAAACCAGAATATAAAGG
ACTCCATCCTCATGGTAAGATTCCAGTTTTAGTTGATGATAATATCACAATTCATGAATCTGCTG
GAATTTGTGCTTATTTAGCCGATCAATATCCTGATAAACAACTTGCTCCCTCTCTTATGAGTCCC
GCAAGAGGCTATTATTATCAATGGTTGTTTTATGCTGCGGTGACGTTAGAACCTCCTGTGGAAC
GATATCTTTTTCATGTTTTCCCTCATTTGTCAGAGAAAGTATTACCTGATAGTGAATATGAAAAC
CTTTCTAAGGACGAAACATTACACTGGTTTGGAAAAGTCTGTCAACCCCTCAATGACCACTTAA
AAGAGAATCAATATCTCGTTGAAAATCAATTTACGGCTGCTGATGTTATTACAGGTGGTGTTTTG
TTTTGGGCGTTCAAAATAGGATTACTAAAAAAGAAACCCCCGTGA

| primer | sequence | length (bp) | Tm (°C) |
|---|---|---|---|
| PCR_GST1478-Forward | 5’ – TTTAGCCGATCAATATCCTG– 3’ | 20 | 54.3 |
| PCR_GST1478-Reverse | 5’ – ACCTGTAATAACATCAGCAG– 3’ | 20 | 54.3 |

**1.4 PCC7418_3557** (801 nucleotides)

ATGTTAGAACTTTATCAATTTGAACTCTCCCAATATAGCGAAAAAGTCCGTTTTCTTCTCGATTA
CAAAGGCTTAGAATACCGTAAAATTGAAGTGACTCCGGGGGTTGGACAAGTGGAAGTCTATCAA
ATGTCTGGACAGCGACAAGTTCCCGTTCTCAAAGATGGGGAAACCGTTGTCGCCGACTCCACTG
AAATCGCCATGTATTTGGAACGCACCTATCCTGAACGTCCCCTGATTCCCACCGCAGCGAAAGA
AAAGGGATTAACCTTATTAATGGAAGAATGGGCGGATGAATCCATTGGCTTAAAAAGTAGAAAA
GCCTTTATGGGGGCGCTAAACCGCAATGAAGCCCTACGCGCTGCGGTCTTACCGCCAGAAACCC
CAGATTTTGTCAGAAGCATTGTCAGTGCGATTCCTTCTGATTTCTTAGACGTTTTAGGAACAGGT
GTCGGCATTGGGGGAGATGCCCTAAAAGCGATTGAAGGTAGCCTCAAGCAAGATTTAGAGGCG
CTGTGTTTAATTTTAGAAGAACAACCCTATCTCACGGGTGCAGTTCCCACCTTGGCTGATTTTAC
TGTGGCAAGTCTGAGTTTATTATTAAAATTCCCAGAAGAATCCTATATGGATATTCCCAGTCAAC
TGGCGGGGAAAGCCCTCCCTGGTCTTGGAGATAACCCTGCGTTTGAACCTTTCTTTACGTGGCG
CGATCGTCTCTATCGAGAATATCGTCAACCCACTGTTCCCAGCAGCCGTAGCGACACCAGCACC
TCTGCACCCAGTTCTATTGAAATTGAGTAA



| primer | sequence | length (bp) | Tm (°C) |
|---|---|---|---|
| PCR_GST3557-Forward | 5’ – CTCAAGCAAGATTTAGAGGC– 3’ | 20 | 56.4 |
| PCR_GST3557-Reverse | 5’ – TTTCAATAGAACTGGGTGCA– 3’ | 20 | 54.3 |

**2. Amino acid sequence alignment of *Halothece* GSTs**

```
PCC7418_0647      MLKLYGATRSRAAIARWYLEELKVPYEFVELDMANGEHRKPPFLAINPMGKVPAIEDNGF
PCC7418_1478      -MKLYYLPLTRASRPHWLLEELEISYELIQVTPDEMSE-KPEYKGLHPHGKIPVLVDDNI
PCC7418_3557      MLELYQFELSQYSEKVRFLLDYKG-LEYRKIEVTPGVG-QVEVYQMSGQRQVPVLKDGET
                   ::**    :: :     * : :   *  ::        :     :    ::*.: *.

PCC7418_0647      SLWESGAILLYLADHYEPEPLTP------QKRAILNQWILFANSTLS-------------
PCC7418_1478      TIHESAGICAYLADQYPDKQLAPSLMS--PARGYYYQWLFYAAVTLEPPVERYLFHVFPH
PCC7418_3557      VVADSTEIAMYLERTYPERPLIPTAAKEKGLTLLMEEWADESIGLKSRKAFMGALNRNEA
                   : :*  *  **   *  . * *             :*   :    .

PCC7418_0647      --IGIFIESNRDNE-------------------------------MPKLFPPLNDHLTQ
PCC7418_1478      LSEKVLPDSEYENLS-----------------------KDETLHWFGKVCQPLNDHLKE
PCC7418_3557      LRAAVLPPETPDFVRSIVSAIPSDFLDVLGTGVGIGGDALKAIEGSLKQDLEALCLILEE
                      ::  .  :                                    : :   .*   * :

PCC7418_0647      HDYLVDDQFSAADVAVG-AYLAYMPRMLQLDFSDYPAIAKYVENLSQRPAFKTGMGF---
PCC7418_1478      NQYLVENQFTAADVITG-GVLFWAFKIGLLKKKPP-------------------------
PCC7418_3557      QPYLTGAVPTLADFTVASLSLLLKFPEESYMDIPSQLAGKALPGLGDNPAFEPFFTWRDR
                  : **.    : **. ..   *

PCC7418_0647      ----------------------------
PCC7418_1478      ----------------------------
PCC7418_3557      LYREYRQPTVPSSRSDTSTSAPSSIEIE
```

Alignment of the amino acid sequences of three *Halothece* GSTs: GST_0647, GST_1478, and GST _3557. The sequences were aligned using ClustalW (www.genome.jp/tools-bin/clustalw). Star denotes conserved amino acid residue.

**3. Amino acid sequence of GSTs for phylogenetic analysis**

**3.1 *Halothece* sp. PCC7418**

**GST_0647**

MLKLYGATRSRAAIARWYLEELKVPYEFVELDMANGEHRKPPFLAINPMGKVPAIEDNGFSLWESGA
ILLYLADHYEPEPLTPQKRAILNQWILFANSTLSIGIFIESNRDNEMPKLFPPLNDHLTQHDYLVDDQF
SAADVAVGAYLAYMPRMLQLDFSDYPAIAKYVENLSQRPAFKTGMGF

**GST_0729**

MQALSWEELENRTNFEIDRVNGPTNAQSRLRLFGRDESEVRVTLYRDHHAWCPYCQKVWLWLEEK
QVPYRVEKVTMFCYGDKERWYKQIVPSGMLPALKLDDRLLTESDDILSQLEQTFGTLGYSMNDRASI
ALRKLERLLFRAWCSWLCVPARSRREDQYNRQQFTDVVSQVEDALQQTPGPYFRDSFSIIDLIFTPF
LERMNASLFYYKGYSLREENPQLGLWFDGMEQRSTYRGTQSDFHTHVHDLPPQMGGCYANDEPQ
TKLNQARVDQGPWLGLPDVMYPEPETSREEALQRVLKHRENLLNVNPASEDLFEEALRCALTNLIT
GEVCSPPAGSASALRYLRDRVSVPRDMSIYAAKRLREALENTASLAGDEQGTPIPVRHRRDQDPANF
AKV

**GST_1478**

MKLYYLPLTRASRPHWLLEELEISYELIQVTPDEMSEKPEYKGLHPHGKIPVLVDDNITIHESAGICAYL
ADQYPDKQLAPSLMSPARGYYYQWLFYAAVTLEPPVERYLFHVFPHLSEKVLPDSEYENLSKDETL
HWFGKVCQPLNDHLKENQYLVENQFTAADVITGGVLFWAFKIGLLKKKPP

**GST_3557**

MLELYQFELSQYSEKVRFLLDYKGLEYRKIEVTPGVGQVEVYQMSGQRQVPVLKDGETVVADSTEIA
MYLERTYPERPLIPTAAKEKGLTLLMEEWADESIGLKSRKAFMGALNRNEALRAAVLPPETPDFVRSI
VSAIPSDFLDVLGTGVGIGGDALKAIEGSLKQDLEALCLILEEQPYLTGAVPTLADFTVASLSLLLKFPE
ESYMDIPSQLAGKALPGLGDNPAFEPFFTWRDRLYREYRQPTVPSSRSDTSTSAPSSIEIE

### 3.2 *Dactylococcopsis salina* PCC8305

**Dacsa_1405**

MLELYQFELSQYSEKVRFLLDYKGLEYRKIEVTPGMGQVEVYQMSGQRQVPVLKDGETVIPDSTDIA
MYLERNYPERPLLPTASREKGLTLLMEEWADESIGLKSRKAFIGALNRNEALRTAVLPSDTPDFVKSIV
SGIPSDLLDALGTGVGIGGEALKAIEGSLKQDLEALCLILQEQPYLTGATPTLADFSVASLSLLLKFPEK
SYMDIPDQLAGKALPGIGDNPAFEPFFSWRDRLYSEYRQATVSTTTSSSSGNAPSSIEIE

**Dacsa_2020**

MLLLQFSTSHYCRKARLALGYKKVKYEVENLTPGFHILKLKPLTGLTTVPALQPTPEPTIGDSTRILHY
LESHYPQPSYTLSNPEQNRYAWLLEDWLDESIGTATRFVYYDWRSKEGKSINPSLSSQLVINIVRRQY
GITPASVKLAKERLQNAIEVLSTWQEKPFLVGESFSVADLAAAALLSPLALIPEYRQEYPWLFQRIAET
HQTCGEPLPPGLD

**Dacsa_2391**

MIKLYGGKRSRASIVQWYLEELSIPYEFVVLDMENGEHKKPDFLAINPMGKVPAIDDNGFYLWESGAI
LSYLSDQYDSEKRSIQERGKINQWILFANATLGPGIFIESNRETEKPKLFPPLNEHLNQYNYLVNDTFT
AADVAVGAYLAYMPMMLQLNFSDYSGIENYVKRLSDRPAFKTSMSR

**Dacsa_2754**

MTIKLYSASVCPFAHRTRLTLLEKGLDFQLIEIDLNNKPDWFSEISPYGKVPVIKHDNNCIWESAIINEYI
DEAFPDISLMPKTASDRAFARIWIDFANTKLVPVFYKMLLEQDPEKQTKWKNQFREHLNFMETEGM
RKLSENGDYWLGDRLSLVDLTFYPWFERFCILEHYRSVFLPKTCSFLQHWWRTMSERDSVQNIKNA
SEFYIAQYQKYANNTVNSVTAQEMRDN

**Dacsa_2853**

MKPLSWEELKTKTNFNLDRVNGNTNSHSRLRLFGQNESEVRVTLYRDHHAWCPYCQKVWLWLEE
KQIPYRIEKVSMFCYGEKERWYKRIVPSGMLPALELDGRLLTESNDILIALEDAFGVLGYSMKDSKVIPL
KKLERQLFRAWCMWLCSGARSSRQEEKNRKQFLDVTEKVETALSETPGAYFLDNFSIVDVLFTPFLE
RMNASLFYYKGYSLREENPHLKQWFAGMEARSTYRGTQSDFHTHVHDLPPQMGGCYANDEPQTK
INQTRVDGGPWLGLPDVGYPEPETSREEALDRVLKHRENLIRVNPMEDQKFDEALRCALSHLITGEL
CQPPAQSASGLRYLRDRINVPRDMSIYAAKRLRESLEQTAALVGEDQGTPIPVQHRRDQDPANFSLT
LSH

**Dacsa_3242**

MKLYDCEAAPSPRKVRLFLAKKGTEVETIQVDLPKGEQFSDWYRQRNPNCTVPALELEEGIVLCESE
AICRYLEEMYPDPILFGRSVIERSAR

### 3.3 *Euhalothece natronophila*

**FRE64_00915**

MLELYQFELSQYSEKVRFLLDYKGLEYRKIEVTPGVGQVEVFQMSGQRQVPVLKDGETVVADSTEIA
MYLDRTYPDRPLVPSSAKERGLSLMMEEWADESIGIKSRKAFIGALNRNEALRAAVLPPDTPDFVKSI
VSGIPSDLLETIGSGVGVGGEALKAAEGSLKQDLDALCLILGEQPYLTGNTPTLADFSVAGLSLLLKFP
EKSFLDLPEQLAGKALPGIGDNPAYEAFFNWRDRLYNDYRQATVSTSSTSASAPSSIEIE

**FRE64_03145**

MEALSWEELEARSNLERDRVNGATNPQARLRLFGHDESEVRVTLYRDHHAWCPYCQKVWLWLEE
KQIPYRIEKVTMFCYGQKERWYKRIVPSGMLPALELDNRLLTESDDILVALEQAFGSLGWSMTDPKV
MSLRKLERLLFRAWCTWLCYPTRNRREEEKNRDQFLKTMQQVEKALSETPSPYFLEDFSVVDVIFT
PYVERMNASLFYYKGYSMREENPYFAKWFDGMETRSTYRGTQSDFHTHAHDLPPQMGGCYANDD
PQTKLNQARVDSGPWMGLPDVNYPEPETSRQEALHRVLKHRQNLIKVNPVSEEIFDPALRCALTHLI
TGEVCPPPAGAATGLRYLRDRVSVPRDMSIYAAKRLRESLEKTASLDSQKQAEPIPVQHRRDQNPAN
FVN



**FRE64_04390**

MTCKLYYHPQSNFARKIRILLMEKKIDYELEAIELSAKPEYFLKISPIGKVPVFVDEDGTVIWDSSLIAEYL
EEKYPHPHLCPQTFQEKIACRKWEEMADTLGDHVIDLWIQGLFNQGKVTRYQSLLQEKISRIIPVFEE
QLKQTKYLLGNETWSMADIAALCSFAYHDLRLNEDWKNKYPHLKNWFNDLHNIESVKLTVPPKKA
GIK

**FRE64_09935**

MIKLYGGKRSRAAIAQWYLEELQVPYEFITLDMENGEHRKPEFLAINPMGKVPAIEDNGFYLWESGAI
LSYLSDQYAKEQSTPQKRAEINQWILFANATLGPGIFIESSRETEKSKLFPPLNDHLSKHDYLVDNQF
TAADVAVGAYLAYMPMMLQLDFSDYPAIANYVKRLSERDGFKASLGSRSN

**FRE64_10100**

MLLLQFSTSHYCRKARLALGYKKINYEVANLTPGLHILKVRPITGLTTVPVLLPTPNNVKSGIGDSTRIF
HYLESHFPEPSYTLAAREQNRYAWLLEDWLDESIGTATRFVYYHWRSNEGKSVNPSLSSQLVINIVR
RQYGITPAAVELAKKRLENAMEVLSPWKEKPFLVGDSLSVADLAAAALLSPLALIPDYKDNYPWLFQ
RVAEIHEQCGEALPPGLEK

**FRE64_11270**

MPTGMLINGEWRKEGYQKDSDGRFLRNPTTFRNWIKADGSSNFLPEVGRYHLYVSLACPWAHRVLI
MRKLKGLEDAISLSIVDPYMGEEGWHFSEEAGTIPDPIFGATYLREIYIKADPNYTGRVTVPVLWDKKT
GTIVNNESRELLRMLDHEFQDIATKKDNYCPPELKSTIEKIIDEIYNPINNGVYRAGFAQSQVAYEEAVT
ELFNALNHWETVLGKQLYLCGEEITEADWCLFATLLRFDAVYYVHFKCNLHRIMDYPNLSRYLLDLY
NQPGVKDTCNFDHIKQHYYRSHPHINPSGIVPVGPAFPLSNTKAASKPHQ

**FRE64_15440**

MKLFYIPLTRATRPRWLLEEMGLSYELVRVGSGEMANKFEYQNLHPHNKVPVLVDDNVTIFESAAIC
SYLADQYPEKELAPSLNSPSRGYYYQWLFYAQTSLEPPVERYIFQVAPDLPEQVLPNSEHTKFSKEEI
FQWFTKVCEPLQRALKNNDYLVDNRLTTVDVVTGGVLYWAYKLGLIKEETPIKKYLMQLIERPAFQR
AHDEINIYKTVA

**3.4 *Thermosynechococcus elongatus***

**tlr0207**

MLKLYGGAKSRASIVRWYLEELGIPYEFVLIDLQAGEQHQPEFLKLNPMGKVPVIVDGDVVLWESGAI
LLYLAQVHGELPKDAAAAAQVYQWVLFANSTLTQAMFPAETRDRQLPPLLKGIETALMGQSYILGK
DFSVADVALGSMLAYLQMLFQVDLSPYPAVADYVARLQQRPAFQKGLMGARA

**3.5 *Rivularia* sp. PCC 7116**

**Riv7116_2251**

MLKFYYNPRSPMARRVWRGLLEKDIPFEGIVMNLNGDQFQPDYLQIHPFHHVPAIDDDGFKMIESIAI
LEYLETKYPNPTLLPKDTQSLATVRMVQMVSTNELVPKVLPLMLEKQDSPKLIAAKEHVEKVLAFFA
DNLKDNSYFGGENLSLADIIVGTDISSLPHLGIDFSKYPNLNKWFEQLMQRPSWQTTEMSPEDFEKF
RRIVTRMVQQKMKP

**Riv7116_2857**

MTTTPLSWQELETLTDYEIDTVNGSTNARARLRLFGQSESDVRVTLYRDNHAWCPYCQKIWLWLE
EKQIPYRIEKVTMFCYGKKESWYKRKVPSGMLPAIELDGQIITESDDILIALERVFGVLNQGMQDSNVI
PLRQLERLLFRAWCSWLCYPASSSQQEQRNREQFIQVVAKIEQALAATPGPYFLDNFGIVDVIFTPY
VERMNASLYYYKGYSMREDNPRFNAWFAAMETRPTYRGTQSDFHTHVHDLPPQMGGCWENGEP
QMLINKARVDNGPWFGLPDVGYPEPENSRSEALQRTIAHRANIIRVNPAKDKLFDEALRCALTHMM
TGKDCVPPAGSDVALRYLRDRINVPRDMSIYAAKHLRESLEKTAALAGERQPEPIPIRHRRDQDPSNF
AIR

**Riv7116_3320**

MLELYQFELSQYSEKVRLILDYKGLEYRKIEVTPGIGQVELFQKTGQRQVPVLKDGNKYIADSTEIAKYI
DAQHPERPLIPQDPKTRGLCLMMEEWADESIGTKSRKALFSAISKDQYLRKALLPNSTPDLLKTLVE
GVPPDILKVLGVGVGYSPDVVQGAMRDLEQDLEALTLILESSPYLLGDEPCLADFAVAGLSVLLKFPD
GNYLDLPDTIKGKGVPGLADNPIYQPFFDWRDRLYVQFRKPIIGSTINSPSAPTSIQID

**Riv7116_3756**

MIKLYGGTFSRASIVHWYLEELEIPYEFIKLDMQAGEHRKPEFLAINPMGKVPAIVDGDYILWESGAILL
YLADKYGKKTLSPQERGIYSQWSLFANATLGPGVFVEATRDKEMPKLMNPLNEILGKQPFLLGNEFT
VADVAVGSMLNYIPMMLKLDLSEYSNVTSYMKKLAERPAFQKVMGSRG



**Riv7116_3957**

METLRLYDFLPSGNGYKIRLLLKQIGMPFERIEINILKGESRTSEFLNKNLNGKIPVLEIGEGKYLAESNAI
LMYLSEGTEFLPYDHYLKAQVLQWLFFEQYSHEPFIATSRYWISILGKAEEYKQALKEKHQRGYAALE
VMENHLTGKNFFVGERYTVADIALFAYTHVADEGGFDLSRFKAIGAWLERIKAQPRFIGIKEG

**Riv7116_4606**

MLKFYYNPISVNARRVWVALLEKQIPFELIRVNLDGDQFDDDFQAINPLGRIPAILDNGLRVVESLAIL
DYLEAKYPTPSLMPSEPSAIAMVSTIKTITVVELQPATIPLSRSLVGLEVEPHKLELAQQRVAIILQMFE
ELLGKQTYFAGEEFTLAEVVAGTLIPSLRLENYPHLKAYTQRLAKRDSWQQTEALPETIEAALPNIREI
LQRRF

**Riv7116_4838**

MKLYYAPASSYSQRVLIALYEKELDFTPIEVNLFDAESREKYLQINRFGKIPTLITDDGEILLEASIIVEYL
DNYQKDIPLIPQDSKANLEMRMLERIIDVYINGGREALFKDSQRSPSPREDKEVVKAKRLLESACNLLD
EKLANRTWLVGDTFTLADCSAAPTLSYLRIVYDYQHLQNLTIYFQRLSEKPSVRKAFGSGREQMKQ
MLSSLKYPVKFEDRL

**Riv7116_5225**

MKLYDLELSGNCYKVRLFLSLLDIKYELVPVDFMSGEHKSPEFLQLNPWGEIPVLEDGDLILRDSQAIL
VYLARKYGGDWFPNDAKNMALVTQWLSTAANEIARGPNDARLNKKFGFAINLDAAQQKAESILNLI
EKHLTTTKNQWLALDYPTIADIACFPYIALAPEGGVMLDKYPAINQWCDRIKKLPNFIEMPGISK

**Riv7116_5707**

MNRILYYHQQSNFSRKIRILLAEKNLDYELKEVNLMDKSAEFLSISPIGKVPVFVEQDGTVIWDSTLIAE
YIDETYPEPSFYPSNPGEKLKCRKWEELADNLGDNIINLWILNFKNNQVPNPYRTRLENSIHRLATVFE
QQLTQTKYLSGNDTWNAADIAALCSFGYYSFRLNEDWLVEYPKIANWFNLLHERESVKSTIPLPLNK
G

**Riv7116_6393**

MSAPVTSPEEKLNQINTQSSSTKANKKGKSLPAGLIIKLGKFVWTTMWQIMMSKLAPSNDKGEYIRP
SSQFRNSINEEENNPYQPCAGRYRLYVGLGCPWAHRTLVVRTLKGLEDCVKVSIVYPSPNEGIWLLN
KPEKNCRTVPELYQVAQPGYQGRSTVPILWDEQTNTIVNNESAEIIVMLNSGLNQFANNPELNLYPE
ELTEEIEKWNEKIYHAVNNGVYRCGFAQTQAAYDQCCDELFSVLDEIDENLENKRYLCGEQLTLADV
RLFTTLFRFDVVYYSLFKCNRRRIVDYKNLGAYLRDLYQLPGVAETCDLESIKQDYYGNLFPLNPGGII
PNGPDISNLKEPSNRENISN

**Riv7116_6557**

MIELYTFTTPNGRKASIMLEEVELPYNVHVIDISKNDQFAPEYVAINPNSKIPAIVDKDTDTTVFESGAIL
MYLADKTGKLLPKEQKSRYQVIEWLMLQMGSIGPMFGQFNHFNLHAPEKIPYAIERYKKETLRLYGV
LDKQLADNEFICGDYSIADVATFPWVTIYEIQEMTLDNHPNLKRWHDTVSKRPAVQRGMKVP

### 3.6 *Acaryochloris marina*

**AM1_0765**

MSTPLSWSELADRTDFHLDPVNGPTNAQSCLRLFGQSEDDVKVTLFRDNHAWCPYCQKIWLWLE
EKQIPYRIEKVTMFCYGEKERWYKQIVPSGMLPALELKGQVITESDDILIALEKEFGPLGKGMQDPAV
MPLRQLERLLFRAWCTWLCYPSRPRQDQRNREQFVSVVKKVEAALSQTPGPYFLEEFGTADAIFTP
YVERMNASLYYYKGYSLREENPRFSDWFDAMESRPTYRGTQSDFHTHAHDLPPQMGGCYKNNDP
QTPINMSRVDNGPWSELPDVTYPEPETSRAEALHRVVKHHENIIKVNPTKDELIDEALRCALTHLITGE
VCTPPSGSDLGLRYLRDRISVPRDMSIYAAKRLKESLEATAALVGNRQGTPIPVRHRRDQDPTNFAKA

**AM1_0859**

MSKFKVYGDIYSGNCYKVKLLLSLLEIEHDWIHIDILKGESRTNDFLERNPNGRVPVLGLPDGRWLFES
NAILHYLAKDSSFLPAEPFAQAQVLQWQFFEQYSHEPYIATSRYIIRYLGSPPDRQADLEARRVWGYA
ALDVMESHLEKQDFFVNTQYSIADISLYAYTHVASEGGFSLKPYTNVRKWLRRVSQHPKHVTMDQF
AP

**AM1_0948**

MISFYYAKPSLFSRPVWITLLEKDLKFEPIYVNMGGDQFTPEFRALNPFCRIPVLVDNGLTITESQAILD
YLDLQYPQPKLLPPSAQAVAKVRQVQMIAVNELVPAIGECLMKKPDQQTYAKHRAVTVLNMFEGL
LEAPYFGGDGLSLADIVTGSLVPVLGDLGFTLDQQPKLQRWLQVLMARPAWQQTQLSAPEKDRF
MRSIRALAKLWQKRRRQRADALLVPKKNTPPTIS



**AM1_1074**

MATYPILYSFRRCPYAMRARLALTVSQQICELREVVLRDKPQEMLDISPKGTVPVLVQVDGSILEESL
EIMMWALKQQDSEVWLRADSGQMAHLHALVAACDGHFKHHLDRYKYAQRYENTNAQEHRAEGS
KFLETLNHQLGETTYLCDQHRSWADMAIAPFVRQFANTDRPWFDAQPWPHLQTWLGEFLESDLF
QQIMGKYPQWKSGEVGPLFPGP

**AM1_1182**

MIELYYWPTPNGHKITLFLEEAGLEYEIKPINIGAGDQFQTDFLKISPNNRIPAIIDQAPADGGEPVSVFE
SGAILLYLAEKTRKFLPNDIRQRNIVQEWLFWQVGGLGPMAGQNHHFSQYAPEKLPYAITRYVNETN

RLYGVLNQHLQGKDFIAGDYSIADMACYPWIAPYKWQGQQLEDFPEINRWFQQIEQRPATVTAYEK
GKQISQSAQLTAEKRKVLFGQTAKTQSSQV

AM1_1230

MIHLYTYTTPNGRKPAILLEELGLPYTLHKVDLGKGEQFSPEFVALNPNSKIPAIKDEDTGVTVFESGAI
LIYLAEKTEKLLPTDAASRAQVMAWLMFQMAGVGPMFGQLGHFRRSAPEPIEYAINRYEQEALRLV
KVLNRQLQERDFIAGEYSIADIATYPWVAAYEYVGLSLDPFPHVQAWLERVGQRPAVQTGMAILTPE
FKSDLAQ

AM1_1608

MPTPEIHLYTASTMNGWKPIIFLEEAKVEYELTYIDFGKKEQKSEWYMRLNPNGRIPTIVDRSNDDFV
VFESGAILWYLAEKYQTFLPIGEKARSEALQWLMFQMSGVGPMMGQAMYFQRIAAPKGNEDPYAI
DRYVTESRRLLEVLDKQLAGKAYLLGDNFTIVDIATYPWARSYPWAKVSIEGLDNLRNWFDRIDARP
ATQKAVTIPKPFPAFFGKGDEATSEAENASRF

AM1_2253

MSSDYTPPKVWQWDSESGGTWAKINRPIAGPTHEQDLPVGKHPLQLYSMATPNGQKVTIILEELLA
LGEAGAEYDAHLIKIGDGDQFGSGFVDVNPNSKIPALVDHSTSTPARVFESGAILLYLAEKFGQLLPTE
HAARTECLSWLFWQMGSAPYLGGGFGHFYSYAPAKIEYCINRFTMEVKRQLDVLNRHLETHSFMA
GDDYSIADIAIWPWYGGVIRNTLYDAAEFIDAPSYTHVVRWAQNIADRPAVQRGRMVNRTWGALSE
QLHERHDAGDFNIKTQDKLNP

AM1_2608

MLTLYQFEPAWGLPNASPFCMKLETYFRMTGLEYQVDTSADVRKAPKGKLPYIEDKGQIIADSNLIIE
YLKTTYGDPLDSHLSPADAAIALAMRRLIEENLYWALVYTRWIDEENWQKTKAVYFSDLPFPLRLLV
PKIARNTVTQNLQGHGMGRHTEAEIYQIAALDIQALSNFLQDKPYFMGEQPTALDASAYSCLANILN
ETLISPLRDKATQLENLVTYCDRMHQTYYA

AM1_2658

MKVYEFKGFPNPARVRIALAEKGLTEAVEFVSVDVPNGEHKQSEFLAKNPSGTVPVLELDDGTTIAEC
TAITEYLDHTSGETTLTGRTPKERAMIHMMQRRAEAGLLDAVGLYFHHATPGLGPDIEAYQCSEWG

EHQRQKAIAGMHYLNDVLAQNTYLAGEQFSMADITAFAGLVFADFAKIEIPAECGHLKAWRERVSQ
RPSVAG

AM1_4752

MTDLTLVIGNKNYSSWSLRAWLFLKQVGVPFQEVRVPLFTDKTRSQLANYSPSGLVPVLITDEGTIW
DSLAICEYGAETHQQGWPQPPAIRAQARAVAAEMHSGFMALRSEMPMNCRARRTGVEPSAVCQT
NIERILSVWQSCRQTYGEAGPWLFGEFSVADAMYAPVASRFVTYGVSLPQIAQDYIHTIFENPHMQE
WLQAGATESEIIQASERGQPISR

AM1_5345

MATSFLSLITFSSIDNAARDKIIECFLSIKNMTQLLPVKKIIFLTLGLISISQSTGQNLAPAIAQNASLIPSE
FTQKQSDLLLYGGPRTRSPLVQWYLEELAVSYQYISLDIRGQEQRQPEFLAINPMGKVPAMVDGTF
KLWESGAILLYLTDKYGKEPQSIEERALLNQWVIFANATLGPGLFREDRREREMPRLLAPLNDIFKQQ
PFILGSELSVADVAVGSYLYYAKLGLSLDFSDYPAVETYLNRLSKRPAFIKTMGQR

AM1_5488

MKLYFMPTTRAVRPRWLLEELNISYKLIRVAMDMSRSKKYGHLHPHGKVPVLIDENVTIFESAAICAY
LADKYIDHGFAPQLDAPARAYYYQWLFYASLTLEAPVEQYMFHVLPGLPNKVLPKQARQTVSPEEA
KQWFAKVCEPLNEQLTTNDYLVEDYFSAADIVTGGVLLWALKLGMLKQESPVKSYLARLMERPAL
QKADEDVYAKVD



AM1_A0001

MKIVSFKICPFVQRVTALLEAKGIDYDIEYIDLSHKPQWFLDLSPNAQVPILITDDDDVLFESDAIVEFL
DEVVGTPLSSDNAVKKAQDRAWSYLATKHYLVQCSAQRSPDAKTLEERSKKLSKAFGKIKVQLGESR
YINGDDLSMVDIAWLPLLHRAAIIEQYSGYDFLEEFPKVKQWQQHLLSTGIAEKSVPEDFEERFTAFY
LAESTCLGQLAKSKNGEACCGTAECTVDDLGCCA

AM1_B0133

MVKAYGFHLSGNSYKVRLLLELLKVDYDWKEMDLVNGEHKSPEYLAVNPLGQVPALVDGETRLTD
AQSILVYLAKQYGGEQWLPTETLPMVQVINWLFTTAGEVRQGPESARLYHFFGVSNINVERTYQKS
EHVLTYLNQHLSTRTWLEFERPTIADVAVFPYVALSRDGKIDLDAYPHILNWIEQVKQLPGFISMPGL

### 3.7 *Halomicronema hongdechloris*

**XM38_010400**

MITLYGHEMSGNSYKVRLLLELLQLDYDWAAVDLMQGEHKSPEYLALNPFGQVPLLIDGDMKLAD
AQAILVYLARQYGGEQWLPMDAVALAQVVRWLSITAGEVRQGPENARLYHLFGATSINIDRAQQKA
DQILTQLDKHLLGRTWLEFQRHTIADIAVFPYVALAPDGQVDLAPYSQVLTWIDRVKHLPGFISMAG
L

**XM38_010430**

MIDLFTYTTPNGRKPSILLEELQLPYTVHAINIGQGEQFSPEFVAINPNSKIPAIVDRDHQLAVFESGAIL
IYLAEKTGKLLPTEAVARAQVMEWLMFQMASVGPMFGQLGHFRNAAPDPIPYAIERYRKETLRLLG
VLDRQLADQPYIAGDYSIADIATFPWVAAVKTPYLDISLADFPWVSGWIDAMKARPAVQVGMNILKP
AFKSDYGTVAPPQETRQALEMAQKQAA

**XM38_018570**

MLTFYYHPLSPVARRVWIALLEKGLPFEARLVQLNGEQWQPEFLALNPFHHVPVLADGELILIESLAI
LDYLEAQYPAPPLTPAKPVALARMRMVQMVVVNELTPHLPALVAESEGIECQPGAALEPGLRFLEQ
QLGNAAYFGGDSLSLADITATCTMSLMQRLGVALADYPALAAWHGRISQRPAWQQSQPEEAALAT
WKRWLALKIKRRQRQLARP

**XM38_028900**

MTDLILTTFDWVPKTPRGYVRDIRVRWALEEARLPYSVTSVPFRDRSAEHFSHQPFGQVPWLTDGDI
SIFESGAILLHLGELSDRLMPAEPHGRSEVIQWLFAALNSVEMASLPWSLFKFSGDTEGTPGRKHLDE
FLKARLHHMEKVLAGRQWLTATFSVADILMADVLRLVDRFDGLVESPACRDYVAHATARPAFVKAH
ADQMAHFAKAD

**XM38_036280**

MLTLYHTPLSLNSRRVWVTLLEKGLHFDTIEMNLSGDQFQPEFLALNPFHHIPVLVDDEVTLIESFAI
MDYLEAKYPIPSLLPSPPTALAKVRMIQMVTVNELLPAISPLTKKMMGFGSPDADALEKAHQQAAV
CLGFCEEKLADWSFFGGDELSLADIVLGTVAPWFDQMELPLDQYPQLQAWIQRLLQRQAWQITQ
PTPEAIDAFKERMAKLMAQRGL

### 3.8 *Prochlorococcus marinus*

**Pro_0130**

MLELYQFEHSAFCLKVRLFLQAKNLQYKVVEITPGIGQINVFKLSGQRQVPVLKDGETIVSDSSEIIQYI
ETITNEPELLPKKPHEAAMAHLIEDWADTTLAKAARLELIKAAAIDPSLRKALLPNDLPNSFKGLIDNL
PCEFMNGLTEVLNQGQSTALLNSLEKLSNSVSSQPWLVGDSLSIADIAVAAQLSLLRFPFSSGESLF
GKGCLGFADNPRLDPLFTWRDQLEKKLIETDPAIL

**Pro_0250**

MSIPPAIVASARMGWKWQWNQLMNGLAPADAEGNYTRTQSQALDSKPPKAEDLLNRSSEDFPLL
VVARSCPWAHRTWLLYELKDLNKSLNILIAKPNPKAGLWKIDPSWKGCKSVLEIYKLCNAPPTHRAT
VPVLVDPKPNNKKTPELLGNESAQLVETLNIWPTEESTPNFYPKELHEEIKDWQELLQDSVNNGVYK
CGFARNQRSYEEACKTLFNSLKIVEKNLSIKGPWLCGEKLTIADIRLFPTMIRWESVYAPLFRCNQSPL
TKFPNLLQWRKNFFNLPKVSKTCDSKNWRNDYFGALFPLNPSNIVPLGPNIQEIINSA

**Pro_0568**

MKEAIAALSWEELTKFAHNQSDLINGPNNSYSLLRLFGQNKSSIRVVFFRDKHAWCPYCQKVWLWL
ELKKIPYAVKKVTMRCYGEKEKWYLKKVPSGLFPAIEIDQELITESDKILLHLEKTFGPLGMQMEHPKII
DLRNLERNLFRSWCIWLCNPSFSKVQSIEREKQFKFIAKEVDNRLSQTNSPWIDPSISNSLESLPGSID
VAFVPYLERMNASLAYYKGIKIRKEFPNIDRWFKSLEILPEYRGTQGDFHTHSHDLPPQMGGCWLDK
NVLQETFSNQIDIGNGLGENETTFEPSTKTLPSAIALTRVLKHREGIKAVNPLGPESFDQPLRAALSYM
ISKQDFIPTQGSAVGLRYLRDRVSVPRDMPLLAAREFRKALEKTAQIDGSEKGAPLPTRHRFDQNPIY
FSKAIDN

**Pro_0786**

MEGIISVENSELRKGSKSILYTFRRCPYAIRARWALFLCGKQVEFREVRLNNKPIELLRASPKGTVPVLIR
ENGQVIDESLEIMHWAIRTSDDNSNKKLLKGFNDKNIKLLIDQNDNSFKFHLDRYKYPNRYEGIEAEE
HRKKAKEILKDWDKRIKYSVNLNLFNDSETIADWSIWPFVRQYRLIDSVRFDKDKELINLRRWLESYLN
SKSYSKIMKKLSFWKSPYDGISTHA

### 3.9 *Gloeocapsa* sp. PCC 7428

**Glo7428_1588**

MLKLYYARPSAYARPVWLALLEKQLPFELISVDLSGEQFEPEFLALNPFSHVPVLVDGDFRVIESLAIL
DYLEARYPEQSLLPTDAIALAKVRMVQMVTLNELLPAVFRLLVRDENSVELEYAQLRAINTLNYFEAL
LEDSPYFAGEQLTLAEIVAGTLVHRMPDLGIALTKYPNLNRWSDRLLARPTWQQIELSPQEWSSFKR
RMRVIPKIWQRRRHQRINALSQQ

**Glo7428_1718**

MLKLYDFPLSGNCHKVRLMLSLLQLDYDLIPVNLKEGEQKSAAFLQLNPLGQVPVLIDDDVVVWDS
QAILVYLARRYGGEKWLPTDADSMSKVMQWLLIAANNIQNSIAAARLHFLFNTQLDLDLAHQKAYQ
ILQIFDEHLSKRDWLECHRLTIADIACFPYIALAPQGKISLDAYPHVTNWINRIKDLPGYISMPGIAG

**Glo7428_2596**

MLKLYGGARSRASIVQWYLEELAVPYEFVLLDMQAGEHRQADFLATNPMGKVPAIVDGDFQLWES
GAILLYLAEKYGKEISSPEERAIAAQWVLFANATLGPGIFVEASRDREMPRLLTPLNEILSRQPFLLGDS
FSVTDVAVGSMLCYIPIMLKLDLSNYPDVLNYMKRLSERPAFQKSIGNRS

**Glo7428_3909**

MIDLYYWTTPNGHKITMFLEEAELPYTLIPVNIGTGDQFKPDFLKIAPNNRIPAIVDRAPADGGEPISVF
ESGAILLYLAEKTGKLIATDIRQRAEVLQWLFWQMGGLGPMAGQNHHFSQYAPEKIPYAIDRYVNET
GRLYAVMNKRLSDRTFLAGNNYSIADIAAYPWIVPYERQGQKLENFPHLQRWFEAIKARPATIRAYEK
AEAFKDQALDIEKSRNLLFNQSANTIQQKS

**Glo7428_4577**

MLELYQFELSQYSEKVRLILDFKGLAYRKIEVTPGVGQLELFRLTGQRQVPVLKDGNQYIADSTQIAK
YLERKYPDRPIIPSDPKQRAMCWLIEEWADESIGIKSRKALFGALTQSESYRKSLLPMATPDVVKTLIG
VVPNDVLKVLGFGVGYGPDVIKSAEEDLKQDLEALCLLLAENPYLVGDQPTLADLAVAGLAMLLKFP
DGPYLELPATLKGKGIPGLGDNIAYQPFFEWRDRLYAQYRKPLTGVSTVGSTPTSIQID

**Glo7428_4724**

MIDLYTFTTPNGRKASIMLEEVQLPYNVHVIDITKDDQFTPEYIAINPNSKIPAIIDQDTGITVFESGAILI
YLAEKTGKLLPTDQKQRFQVLEWLMLQMGSVGPMFGQLNHFKKFAPKEIPYAIQRYEKETLRLYGV
LDQQLANNEFLCGDYSIADIATYPWVAIYEFQGLTLDNHPYLKRWVETMQQRPAVQRGMSVP

**Glo7428_4988**

MIVVHHLNNSRSQRILWLLEELELNYEIKRYERKPKTMLAPESLREVHPLGKSPVITDEALTLAESGAII
EYLVERYGKGRFVPPPGTAERLRYTYWLHYAEGSAMPLLLLKLVFDRIEQQAPFFVKPMAQLIANQT
KSSFIEPRIKQHLNYLEAELGKSLWFAGEEFTAADVQMSFPIEVAVSRAGLDASYPKLIDFLERIHARPA
YQRALERGGTYELLS

**3.10 *Pleurocapsa* sp. PCC 7327**

**Ple7327_1183**

MKSSKKRKSLPPKAIIKLGRFVWTSLWHLMMSNLAPRSQSGEYVRPASAFRNSVGTEPENPYQPAA
GRYCLYVGWGCPWAHRTLIVRTIKGLEAAIPVTIVSPAPEEGGWAFEKPEEGCRTLAEFYQKAQPGYE
GRCTVPVLFDRQTKTIVNNESAEIIVMLNSQFNKWATNPALDLYPEELKEKIDWWNEKIYSAVNNGV
YRCGFAQTQEAYEKACNELFAVLDEIDAVLAGSRYLCGDRVTLADVRLFTTLFRFDIVYYGLFKCNRK
RIRDYTNLGGYLCDLYQLPGVADTCNLEAVKREYYGNLFPLNPGGIIPIGPEITNLLEPHNRERVGVSV
N



**Ple7327_2086**

MLKLYGGVRSRASIVQWYLEELGIPYEFVLLDMEAGEHRKPDFLAINPIGKVPAIVEGDFRLWESGAIL
LYLAEKYGKMPESLEGKSTIAQWVIFANSTLATGLFVESVREQETPKLLTPLNQIFDRQPFLLGDEFT
VADVAVGSILAYVPMMLKLDLSEYPAVLGYIQRISERPAFGKTIGKRSA

**Ple7327_2157**

MLELYQFELSQYSEKVRLILDYKGLEYKKIEVTPGIGQLELFRLSGQRQVPVLKDGETFIADSTEIAFYL
DRKYPEKPIIPTEPLLRGQCLLIEEWADESIGLKGRKAFIGALNQNQNFRVSILPKNVPDFFKSLVGAVP
SEFLGLLGTGVGFGPDAIKEARRGLEQDLEALTLILQNRPYLVGDEPTLADLAVAGLSTILKFPAGNYL
NVPEQLKGKGIPGLADRSAYEPFFSWRDRLYAEYRKPLTGSGATDSSPTSIEID

**Ple7327_2576**

MAMSNSLDRAPETLHFYFNRNCPYAQRSWIALIELGIAYEPIEIELGKDNKTDWFRALNPNGTVPTIK
HGETVVYESLVVNEYLCEVFGGDLMPSTPANRARARILMSRCDAKFVKLGYSYLSHKRREDETKDDQ
LRSQLEEELRFLDNAIGNWGGSYFLGDTLTLADIAFIPFFQRMNVALASFKNFKLENLNLPHLNAWL
EAISHRDSCSQTQMSAQQIEEVYARFLNLDYFKRIGIAS

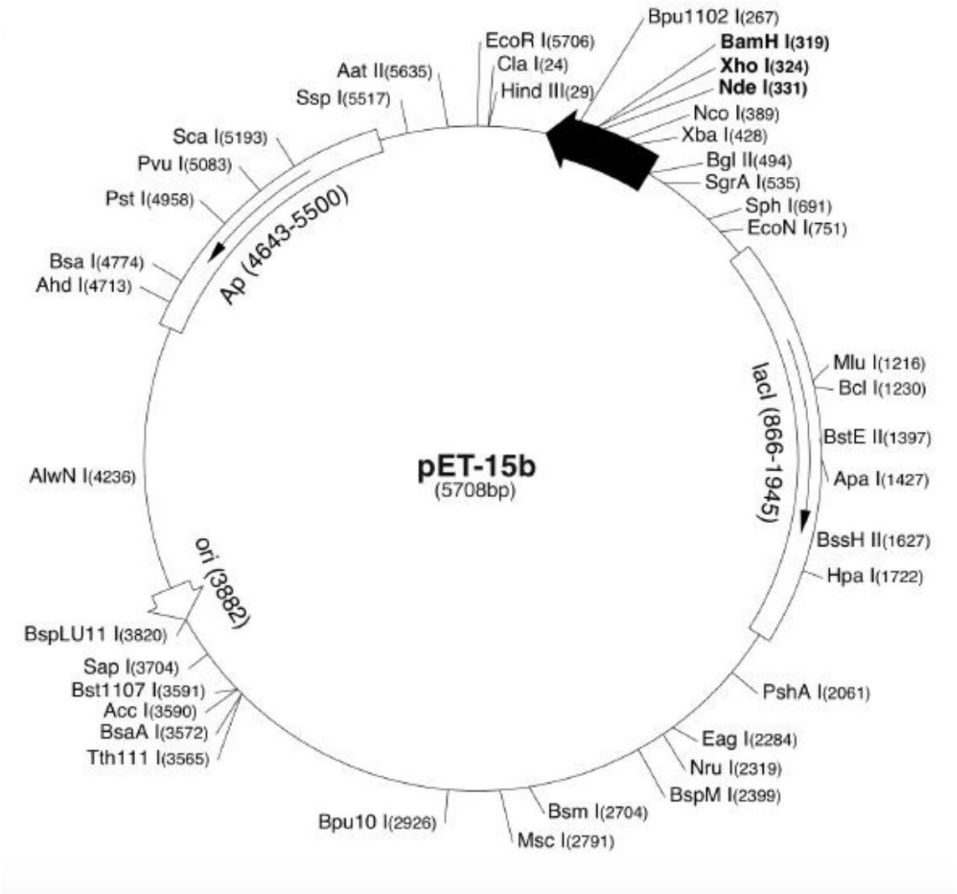
**3.11 *E. coli* K12**

**GST-A**

MKLFYKPGACSLASHITLRESGKDFTLVSVDLMKKRLENGDDYFAVNPKGQVPALLLDDGTLLTEGV
AIMQYLADSVPDRQLLAPVNSISRYKTIEWLNYIATELHKGFTPLFRPDTPEEYKPTVRAQLEKKLQY
VNEALKDEHWICGQRFTIADAYLFTVLRWAYAVKLNLEGLEHIAAFMQRMAERPEVQDALSAEGLK

**4. Plasmid vector map**

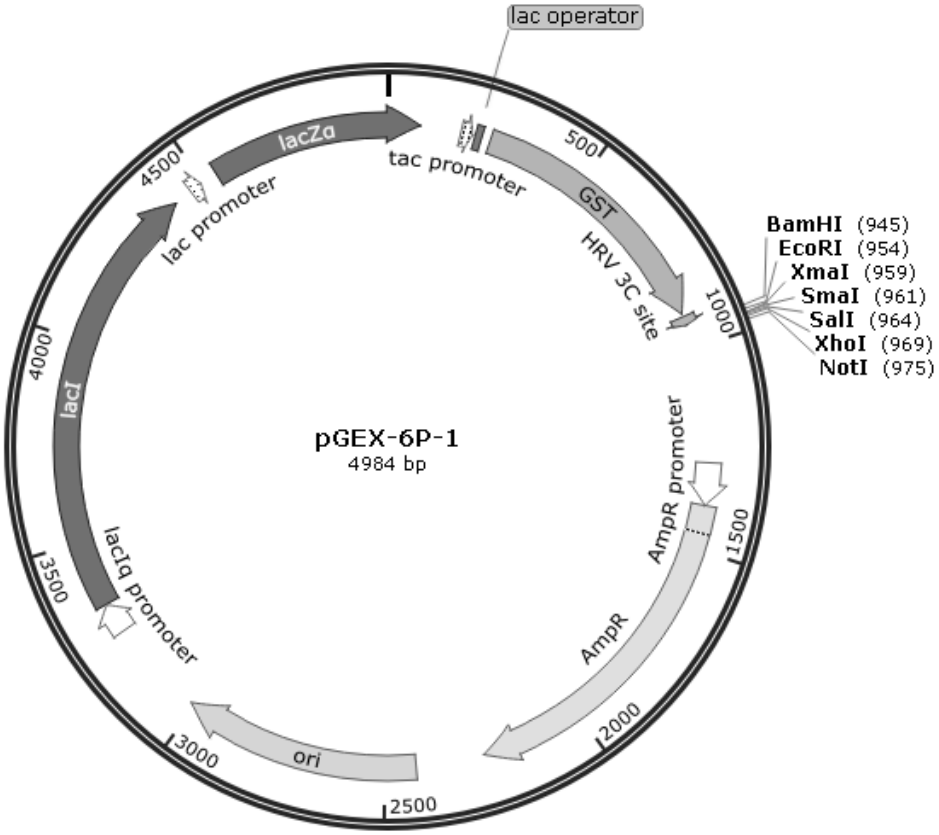
**4.1 pET15b vector (Invitrogen, USA)**






picture source: https://www.addgene.org/vector-database/2543/

**4.2 pGEX6P-1 vector (GE-Healthcare, USA)**






picture source: https://www.addgene.org/78712/

**5. Multiple sequence alignment of pET15b_3557 and other orthologs**

```
PCC7418_3557      MLELYQFELSQYSEKVRFLLDYKGLEYRKIEVTPGVGQVEVYQMSGQRQVPVLKDGETVV
Dacsa_1405        MLELYQFELSQYSEKVRFLLDYKGLEYRKIEVTPGMGQVEVYQMSGQRQVPVLKDGETVI
Fre64_00915       MLELYQFELSQYSEKVRFLLDYKGLEYRKIEVTPGVGQVEVFQMSGQRQVPVLKDGETVV
Glo7428_4577      MLELYQFELSQYSEKVRLILDFKGLAYRKIEVTPGVGQLELFRLTGQRQVPVLKDGNQYI
Riv7116_3320      MLELYQFELSQYSEKVRLILDYKGLEYRKIEVTPGIGQVELFQKTGQRQVPVLKDGNKYI
Ple7327_2157      MLELYQFELSQYSEKVRLILDYKGLEYKKIEVTPGIGQLELFRLSGQRQVPVLKDGETFI
                  ****************::**:*** *:*******:**:*::: :***********:  :

PCC7418_3557      ADSTEIAMYLERTYPERPLIPTAAKEKGLTLLMEEWADESIGLKSRKAFMGALNRNEALR
Dacsa_1405        PDSTDIAMYLERNYPERPLLPTASREKGLTLLMEEWADESIGLKSRKAFIGALNRNEALR
Fre64_00915       ADSTEIAMYLDRTYPDRPLVPSSAKERGLSLMMEEWADESIGIKSRKAFIGALNRNEALR
Glo7428_4577      ADSTQIAKYLERKYPDRPIIPSDPKQRAMCWLIEEWADESIGIKSRKALFGALTQSESYR
Riv7116_3320      ADSTEIAKYIDAQHPERPLIPQDPKTRGLCLMMEEWADESIGTKSRKALFSAISKDQYLR
Ple7327_2157      ADSTEIAFYLDRKYPEKPIIPTEPLLRGQCLLIEEWADESIGLKGRKAFIGALNQNQNFR
                  .***:** *::  :*::*::*  .  :.   ::********** *.***::.*:.:.:  *

PCC7418_3557      AAVLPPETPDFVRSIVSAIPSDFLDVLGTGVGIGGDALKAIEGSLKQDLEALCLILEEQP
Dacsa_1405        TAVLPSDTPDFVKSIVSGIPSDLLDALGTGVGIGGEALKAIEGSLKQDLEALCLILQEQP
Fre64_00915       AAVLPPDTPDFVKSIVSGIPSDLLETIGSGVGVGGEALKAAEGSLKQDLDALCLILGEQP
Glo7428_4577      KSLLPMATPDVVKTLIGVVPNDVLKVLGFGVGYGPDVIKSAEEDLKQDLEALCLLLAENP
Riv7116_3320      KALLPNSTPDLLKTLVEGVPPDILKVLGVGVGYSPDVVQGAMRDLEQDLEALTLILESSP
Ple7327_2157      VSILPKNVPDFFKSLVGAVPSEFLGLLGTGVGFGPDAIKEARRGLEQDLEALTLILQNRP
                   ::**  .**..::::  :* :.*  :* *** . :.::    .*:***:** *:* . *

PCC7418_3557      YLTGAVPTLADFTVASLSLLLKFPEESYMDIPSQLAGKALPGLGDNPAFEPFFTWRDRLY
Dacsa_1405        YLTGATPTLADFSVASLSLLLKFPEKSYMDIPDQLAGKALPGIGDNPAFEPFFSWRDRLY
Fre64_00915       YLTGNTPTLADFSVAGLSLLLKFPEKSFLDLPEQLAGKALPGIGDNPAYEAFFNWRDRLY
Glo7428_4577      YLVGDQPTLADLAVAGLAMLLKFPDGPYLELPATLKGKGIPGLGDNIAYQPFFEWRDRLY
Riv7116_3320      YLLGDEPCLADFAVAGLSVLLKFPDGNYLDLPDTIKGKGVPGLADNPIYQPFFDWRDRLY
Ple7327_2157      YLVGDEPTLADLAVAGLSTILKFPAGNYLNVPEQLKGKGIPGLADRSAYEPFFSWRDRLY
                  ** *  * ***::**.*: :****   ::::*  : **.:**:.*.  ::.** ******

PCC7418_3557      REYRQPTVPSSRSDTSTSAPSSIEIE
Dacsa_1405        SEYRQATVSTTTSSSSGNAPSSIEIE
Fre64_00915       NDYRQATVS--TSSTSASAPSSIEIE
Glo7428_4577      AQYRKPLTGVSTVG---STPTSIQID
Riv7116_3320      VQFRKPIIGSTINSP--SAPTSIQID
Ple7327_2157      AEYRKPLTGSGATD---SSPTSIEID
                   ::*:.       .   .:*:**:*:
```



Multiple amino acid sequence alignment of putative PCC7418_3557 GST and other six closet cyanobacterial orthologs: *D. salina* (Dacsa_1405), *Euhalothece natronophila* (Fre64_00915), *Rivularia* sp. (Riv7116_3220), *Gloeocapsa* sp. (Glo7428_4577) and *Pleurocapsa* sp. (Ple7327_2157). The amino acid sequences were searched from KEGG database. The substitution site was labeled as green. For * means conserved in all aligned GSTs, while : and . means partial conserved in some aligned GSTs.

## 6. Nucleotide sequencing and sequence alignment

sequence alignment: pET15b_0647 and putative PCC7418_0647

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 1020 bits(552) | 0.0 | 552/552(100%) | 0/552(0%) | Plus/Plus |

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Query  52   ATGCTTAAACTATATGGTGCAACCAGAAGTCGCGCCGCGATCGCGCGATGGTATTTAGAA  111
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Sbjct  1    ATGCTTAAACTATATGGTGCAACCAGAAGTCGCGCCGCGATCGCGCGATGGTATTTAGAA  60

Query  112  GAACTGAAAGTTCCCTACGAATTTGTTGAACTGGATATGGCAAATGGGGAACATCGCAAA  171
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Sbjct  61   GAACTGAAAGTTCCCTACGAATTTGTTGAACTGGATATGGCAAATGGGGAACATCGCAAA  120

Query  172  CCACCATTTCTTGCTATTAACCCCATGGGAAAAGTTCCCGCGATTGAAGATAATGGCTTT  231
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Sbjct  121  CCACCATTTCTTGCTATTAACCCCATGGGAAAAGTTCCCGCGATTGAAGATAATGGCTTT  180

Query  232  TCTTTATGGGAGTCGGGAGCAATTCTTTTATATTTAGCCGATCACTACGAACCCGAACCA  291
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Sbjct  181  TCTTTATGGGAGTCGGGAGCAATTCTTTTATATTTAGCCGATCACTACGAACCCGAACCA  240

Query  292  CTAACTCCACAAAAACGGGCAATTCTGAATCAATGGATTTTATTTGCGAATTCAACCCTT  351
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Query  352  AGCATTGGTATTTTTATCGAGAGTAACCGCGATAATGAAATGCCAAAACTCTTTCCCCCC  411
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Sbjct  361  TTAAACGATCATTTAACCCAACACGACTACTTAGTTGATGATCAATTTAGTGCTGCTGAT  420

Query  472  GTTGCTGTCGGGGCTTATTTAGCTTATATGCCCAGAATGTTACAACTGGATTTTTCCGAC  531
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Query  532  TATCCTGCTATTGCTAAATATGTGGAAAATCTCTCCCAACGTCCTGCATTTAAAACAGGA  591
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Sbjct  541  ATGGGCTTCTAA  552
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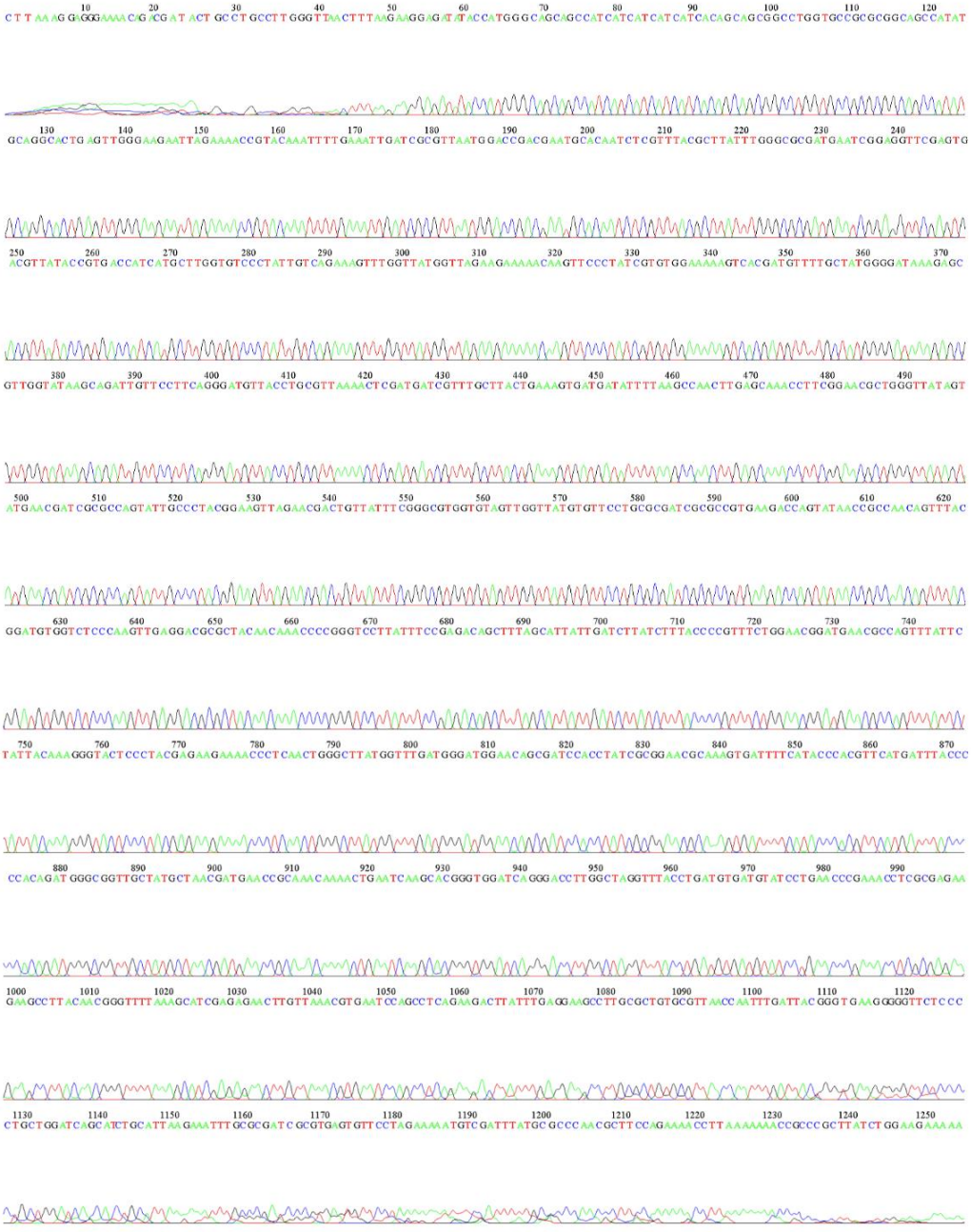
pET15b_0729



sequence alignment: pET15b_0729 and putative PCC7418_0729

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 1251 bits(677) | 0.0 | 677/677(100%) | 0/677(0%) | Plus/Plus |

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Sbjct  1    ATGCAGGCACTGAGTTGGGAAGAATTAGAAAACCGTACAAATTTTGAAATTGATCGCGTT  60

Query  124  AATGGACCGACGAATGCACAATCTCGTTTACGCTTATTTGGGCGCGATGAATCGGAGGTT  183
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Sbjct  61   AATGGACCGACGAATGCACAATCTCGTTTACGCTTATTTGGGCGCGATGAATCGGAGGTT  120

Query  184  CGAGTGACGTTATACCGTGACCATCATGCTTGGTGTCCCTATTGTCAGAAAGTTTGGTTA  243
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Sbjct  121  CGAGTGACGTTATACCGTGACCATCATGCTTGGTGTCCCTATTGTCAGAAAGTTTGGTTA  180

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Sbjct  181  TGGTTAGAAGAAAAACAAGTTCCCTATCGTGTGGAAAAAGTCACGATGTTTTGCTATGGG  240

Query  304  GATAAAGAGCGTTGGTATAAGCAGATTGTTCCTTCAGGGATGTTACCTGCGTTAAAACTC  363
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Sbjct  241  GATAAAGAGCGTTGGTATAAGCAGATTGTTCCTTCAGGGATGTTACCTGCGTTAAAACTC  300

Query  364  GATGATCGTTTGCTTACTGAAAGTGATGATATTTTAAGCCAACTTGAGCAAACCTTCGGA  423
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Query  424  ACGCTGGGTTATAGTATGAACGATCGCGCCAGTATTGCCCTACGGAAGTTAGAACGACTG  483
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Sbjct  361  ACGCTGGGTTATAGTATGAACGATCGCGCCAGTATTGCCCTACGGAAGTTAGAACGACTG  420

Query  484  TTATTTCGGGCGTGGTGTAGTTGGTTATGTGTTCCTGCGCGATCGCGCCGTGAAGACCAG  543
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Sbjct  421  TTATTTCGGGCGTGGTGTAGTTGGTTATGTGTTCCTGCGCGATCGCGCCGTGAAGACCAG  480

Query  544  TATAACCGCCAACAGTTTACGGATGTGGTCTCCCAAGTTGAGGACGCGCTACAACAAACC  603
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  TATAACCGCCAACAGTTTACGGATGTGGTCTCCCAAGTTGAGGACGCGCTACAACAAACC  540

Query  604  CCGGGTCCTTATTTCCGAGACAGCTTTAGCATTATTGATCTTATCTTTACCCCGTTTCTG  663
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  CCGGGTCCTTATTTCCGAGACAGCTTTAGCATTATTGATCTTATCTTTACCCCGTTTCTG  600

Query  664  GAACGGATGAACGCCAGTTTATTCTATTACAAAGGGTACTCCCTACGAGAAGAAAACCCT  723
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601  GAACGGATGAACGCCAGTTTATTCTATTACAAAGGGTACTCCCTACGAGAAGAAAACCCT  660

Query  724  CAACTGGGCTTATGGTT  740
            |||||||||||||||||
Sbjct  661  CAACTGGGCTTATGGTT  677
```

pET15b_1478

sequence alignment: pET15b_1478 and putative PCC7418_1478

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 1031 bits(558) | 0.0 | 561/562(99%) | 1/562(0%) | Plus/Plus |

```
Query  29   ATGAAACTTTATTATCTTCCGTTAACCCGAGCCAGTCGCCCTCATTGGCTATTAGAAGAA  88
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    ATGAAACTTTATTATCTTCCGTTAACCCGAGCCAGTCGCCCTCATTGGCTATTAGAAGAA  60

Query  89   CTGGAAATTTCCTATGAATTAATTCAAGTGACCCCTGATGAAATGTCGGAGAAACCAGAA  148
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CTGGAAATTTCCTATGAATTAATTCAAGTGACCCCTGATGAAATGTCGGAGAAACCAGAA  120

Query  149  TATAAAGGACTCCATCCTCATGGTAAGATTCCAGTTTTAGTTGATGATAATATCACAATT  208
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TATAAAGGACTCCATCCTCATGGTAAGATTCCAGTTTTAGTTGATGATAATATCACAATT  180

Query  209  CATGAATCTGCTGGAATTTGTGCTTATTTAGCCGATCAATATCCTGATAAACAACTTGCT  268
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  CATGAATCTGCTGGAATTTGTGCTTATTTAGCCGATCAATATCCTGATAAACAACTTGCT  240

Query  269  CCCTCTCTTATGAGTCCCGCAAGAGGCTATTATTATCAATGGTTGTTTTATGCTGCGGTG  328
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  CCCTCTCTTATGAGTCCCGCAAGAGGCTATTATTATCAATGGTTGTTTTATGCTGCGGTG  300

Query  329  ACGTTAGAACCTCCTGTGGAACGATATCTTTTTCATGTTTTCCCTCATTTGTCAGAGAAA  388
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  ACGTTAGAACCTCCTGTGGAACGATATCTTTTTCATGTTTTCCCTCATTTGTCAGAGAAA  360

Query  389  GTATTACCTGATAGTGAATATGAAAACCTTTCTAAGGACGAAACATTACACTGGTTTGGA  448
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GTATTACCTGATAGTGAATATGAAAACCTTTCTAAGGACGAAACATTACACTGGTTTGGA  420

Query  449  AAAGTCTGTCAACCCCTCAATGACCACTTAAAAGAGAATCAATATCTCGTTGAAAATCAA  508
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  AAAGTCTGTCAACCCCTCAATGACCACTTAAAAGAGAATCAATATCTCGTTGAAAATCAA  480

Query  509  TTTACGGCTGCTGATGTTATTACAGGTGGTGTTTTGTTTTGGGCGTTCAAAATAGGATTA  568
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  TTTACGGCTGCTGATGTTATTACAGGTGGTGTTTTGTTTTGGGCGTTCAAAATAGGATTA  540

Query  569  CTaaaaaaaGAAACCCCCGTGA  590
            || ||||||||||||||||||
Sbjct  541  CT-AAAAAAGAAACCCCCGTGA  561
```

pET15b_3557

sequence alignment: pET15b_3557 and putative PCC7418_3557

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 1055 bits(571) | 0.0 | 573/574(99%) | 0/574(0%) | Plus/Plus |

```
Query  44   ATGTTAGAACTTTATCAATTTGAACTCTCCCAATATAGCGAAAAAGTCCGTTTTCTTCTC  103
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    ATGTTAGAACTTTATCAATTTGAACTCTCCCAATATAGCGAAAAAGTCCGTTTTCTTCTC  60

Query  104  GATTACAAAGGCTTAGAATACCGTAAAATTGAAGTGACTCCGGGGGGTTGGACAAGTGGAA  163
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GATTACAAAGGCTTAGAATACCGTAAAATTGAAGTGACTCCGGGGGGTTGGACAAGTGGAA  120

Query  164  GTCTATCAAATGTCTGGACAGCGACAAGTTCCCGTTCTCAAAGATGGGGAAACCGTTGTC  223
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  GTCTATCAAATGTCTGGACAGCGACAAGTTCCCGTTCTCAAAGATGGGGAAACCGTTGTC  180

Query  224  GCCGACTCCACTGAAATCGCCATGTATTTGGAACGCACCTATCCTGAACGTCCCCTGATT  283
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  GCCGACTCCACTGAAATCGCCATGTATTTGGAACGCACCTATCCTGAACGTCCCCTGATT  240

Query  284  CCCACCGCAGCGAAAGAAAAGGGATTAACCTTATTAATGGAAGAATGGGCGGATGAATCC  343
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  CCCACCGCAGCGAAAGAAAAGGGATTAACCTTATTAATGGAAGAATGGGCGGATGAATCC  300

Query  344  ATTGGCTTAAAAAGTAGAAAAGCCTTTATGGGGGCGCTAAACCGCAATGAAGCCCTACGC  403
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  ATTGGCTTAAAAAGTAGAAAAGCCTTTATGGGGGCGCTAAACCGCAATGAAGCCCTACGC  360

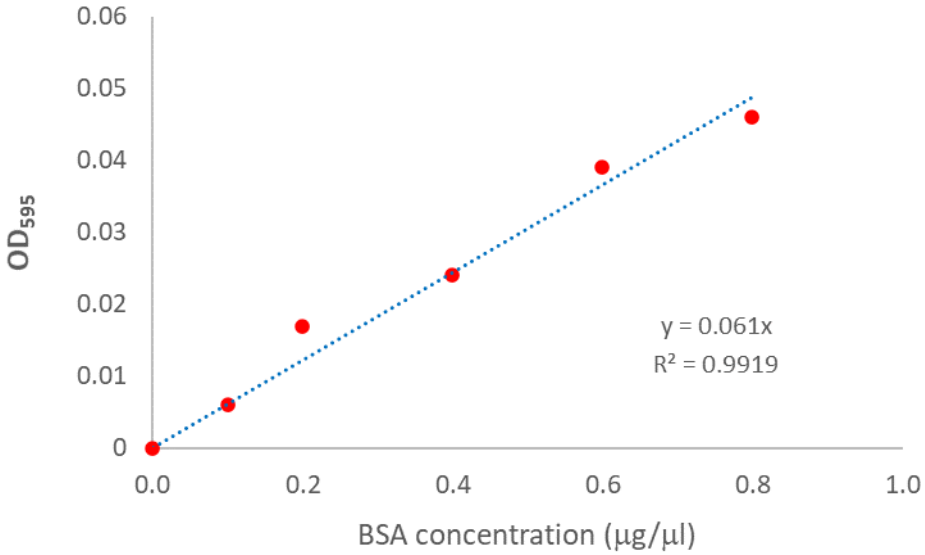
Query  404  GCTGCGGTCTTACCGCCAGAAACCCCAGATTTTGTCAGAAGCATTGTCAGTGCGATTCCT  463
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GCTGCGGTCTTACCGCCAGAAACCCCAGATTTTGTCAGAAGCATTGTCAGTGCGATTCCT  420

Query  464  TCTGATTTCTTAGACGTTTTAGGAATAGGTGTCGGCATTGGGGGAGATGCCCTAAAAGCG  523
            |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  421  TCTGATTTCTTAGACGTTTTAGGAACAGGTGTCGGCATTGGGGGAGATGCCCTAAAAGCG  480

Query  524  ATTGAAGGTAGCCTCAAGCAAGATTTAGAGGCGCTGTGTTTAATTTTAGAAGAACAACCC  583
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  ATTGAAGGTAGCCTCAAGCAAGATTTAGAGGCGCTGTGTTTAATTTTAGAAGAACAACCC  540

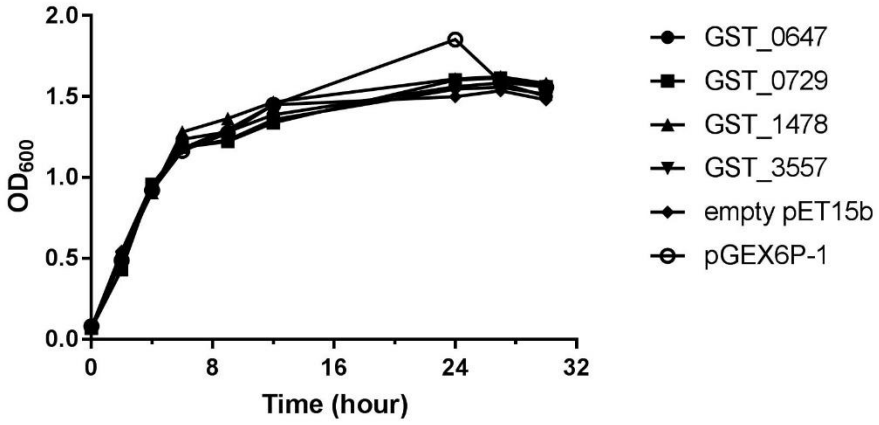
Query  584  TATCTCACGGGTGCAGTTCCCACCTTGGCTGATT  617
            ||||||||||||||||||||||||||||||||
Sbjct  541  TATCTCACGGGTGCAGTTCCCACCTTGGCTGATT  574
```

7. Protein standard curve

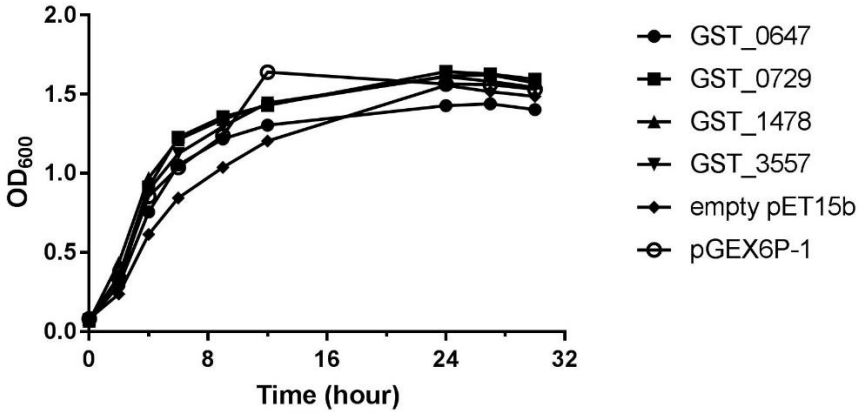


8. Growth of *E. coli* BL21 containing empty pET15b vector

Normal condition, LB-broth without IPTG




Normal condition, LB-broth with 0.5 mM IPTG



# VITA

| | |
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