CHAPTER 4

DISCUSSION AND CONCLUSION

4.1) DISCUSSION

4.1.1) INVESTIGATION OF PROTEIN EXPRESSION IN COLD SHOCK AT DIFFERENT TEMPERATURES

4.1.1.1 Growth profile of *B. stearothermophilus* TLS33 under cold shock

B. stearothermophilus TLS33 was studied by Sinchaikul S. *et al* and Sookkheo B. *et al* (56-57). From previous study, this thermophile can be grown well in 65°C. Although it can grow well in high temperature, it is also stored in lower temperature. Nevertheless, it seems like its growth rate was decreased. The previous study mentioned that it would enter to its sporulation (115). In general, *Bacillus* sp. will construct its spore for keeping their genetic materials for longer. The spore of *B. stearothermophilus* was also utilized in sterilization food test. That meant the spore of this species could be durable in heat condition. In this study, the proteome of this bacterium has been analyzed when this bacterium was under lower its growth optimum temperature (65°C) i.e. 37°C and 25°C. In rationale, the completed genome sequencing *Bacillus* sp. mesophile, *Bacillus subtilis* can grow well at 37°C (117) has been studied on the cold shock response for years. Thus this temperature was choosen for studied to induce interesting proteins in *B. stearothermophilus* TLS33 when it was under cold stress. Also, it is well know that 25°C is room temperature. The growth

rate of this bacterium at 37°C and 25°C were slow down after it has been encountered cold shock stress within 2 hours.

4.1.1.2 2DE, protein identification and protein function

The figure 3.2 demonstrated 2DE gel of cell extract from *B. stearothermophilus* TLS33. In general, the protein spots have to digest with proteolytic enzyme such as trypsin in order to specific cleavage the interesting proteins and finally we can obtain digested peptide. The individual digested peptide will analyzed their mass by any mass spectrometry method.

The 53 spots of total proteins from cell extracts in three temperatures were tryptic digested and searched against MS-FIT and MASCOT, which are general protein identifying software in proteomic work. So, SubtiList database (http://genolist.pasteur.fr/subtilist) supplementing with EMBL/GenBank/DDBJ databases was used for searching protein functions. Interestingly, there were eight protein spots were differential expressed under temperatures of 37°C and 25°C whereby, the eight spots were postulated that they are involved in sporulation signaling pathway. In figure 3.5, the correlation of cold induced proteins was shown. In figure 3.4, protein synthesis was analyzed by computer software aid. The software was able to indicate protein spot volume of 2DE gel images. These eight spot volumes were generated by this software as well. TagE (Glucosyl transferase), RsbT (Anti-Sigma B factor), MrpA (MrpA protein homolg) PyrC (dihydroorthase), YbbB (Hypothetical transcriptional regulator intergenic region) RibT protein, CysH (Phosphoadenosine phosphosulfate reductase) including RsfA (prespore specific transcriptional activator), have been postulated to involve in signaling pathway in

sporulation in gram-positive bacteria. However, the sporulation pathway, where is available on internet or bioinformatics, could provide protein function as well as their interaction and correlation to other proteins. In this case, the diagram which can demonstrate correlation and prediction protein expression was generated. The activation and deactivation of signal pathway to maintain the sporulation of *B. stearothermophilus* TLS33 under cold shock was proposed in figure 3.5. TagE was induced by 25°C cold stress but not in 37°C. In production of Sigma factor, TagE route would be activated in forspore via RsbW/σ^B route. Alternatively, it will activate the PAS-RsbP gene and this route is only activated when bacterium is experiencing shock at 37°C or lower. There has also been reported that glucosyltransferase is an induced-cold response enzyme that functions in transcription responsed by Pho~P under phosphate starvation conditions whereas its stress regulon is under control of the alternative transcription factor σ^B (75,77). For activation YbbB route, it is similar to TagE, YbbB was induced by cold stress and finally it could produce σ^F/σ^G. Also, RsfA also activates σ^F/σ^G in forespore leads to transcription of spoIIR and spoIIQ immediately after asymmetric division, and of several genes that can be disrupted without preventing formation of stress resistant spores (74,78). For RibT protein, PsbT was induced at only 37°C, eventhough there was no clearly established that this protein involved in production of σ^F/σ^G in the forespore. However, there has been additional studies reported that RsbT is a protein involved in expression of σ^B factor that controls the general stress response in vegetative cell early sporulation of *Bacillus* sp. (79,83). However, MrpA and PyrC seemed to be down-regulated when bacteria were at 37°C or lower. Mrp functions multi-resistance and pH homeostasis as Na^+/H^+

antiporter that affect post-translational regulation control of σ^H in the early sporulation of cell cycle (89, 90). Finally, the activation and deactivation of cold shock-induced proteins correlating to the σ^F/σ^G production in forespore are summarized in figure 3.6. The down regulation of MrpA and PyrC were also observed when temperature was dropped below 37°C. However, the proteins and their related protein in the TageE, YbbB, RsfA, MrpA and PyrC routed at 65°C could be not identified. First, the failure might due to the limitation of 2DE and result in the related protein could not be observed. Second, MALDI-TOF MS failed to indetify the proteins which caused by post-translational modification of related proteins. Third, TagE, YbbB and RsfA probably have been turned on at 37°C while MrpA and PyrC were turned off. If the third argument is valid, it is necessary to find the unknown proteins which cause these regulations upstream of the signaling pathway. However, the other protein which involved in forespspore production could not identify. These could be suggested that first post-translational modification could effect to experimental tryptic digested peptide. Whereby, their mass spectrums probably do not match to theoretical tryptic digested peptide mass, because the post-translational modified peptides would be different from theoretical peptide mass. Second, since *B. stearothermophilus* TLS33 is incompleted genome sequencing bacterium. Thus, a number of proteins probably could not be identified. Third, since limitation of 2DE, such as protein solubility, hydrophobicity of protein including high acidic or basic proteins, this might because 2DE missed a number of proteins. However, the correlation of cold-induced proteins was summarized in figure 3.6.

Nevertheless, some of interesting proteins were still observed. However, non-gel proteomic approach such as isotope-coded affinity tags (ICAT) or iTRAQ™ seem to be alternative methodology that might aid this study to elucidate differential protein expression in different cold shock stress. So far, 2DE is traditional method for protein complex separation.

4.1.2) STRESS RESPONSES OF *BACILLUS STEAROTHERMOPHILUS* TLS33

4.1.2.1 Growth profile in individual conditions

In this study, *B. stearothermophilus* TLS33 was challenged to its unfavour conditions such as 10% w/v ethanol, 10% w/v salt stress and 25°C cold stress. In order to investigate important proteins controlling adaptation of this thermophile to stress condition, 2DE, powerful method for protein separation was utilized in this study. The aim of this study is to understand to adaptation thermophile bacterium, *B. strearothermophilus* TLS33, in proteome level. It was found that there are many proteins that were globally changed to protein expression. 10% w/v NaCl, 10% v/v ethanol and 25°C cold stress are the stress conditions that have been use for stress this thermophile to investigate the changes of its proteome. In figure 3.7 when bacterium was challenged to salt stress at mid-exponential phase, it was observed that stopped the growth of cell number. Likewise, other stress conditions, ethanol stress, the cell number was reduced in first 2 hours and there was no more decreasing after 2 hours. Nevertheless, there was a slight increase of the growth rate which would not neither increasing nor decreasing in the late of stress conditions. Therefore, it could be

concluded that the bacterium has adapted to stress conditions by keeping its cell number to no more decreasing. At this point, we might assume that there might be changed in proteome level of *B. stearothermophilus* TLS33. Therefore, its proteome was studied in the first 2 hours.

4.1.2.2 2DE, Image analysis and Protein identification

In generally, 2DE gel images have been analyzed with image analysis scientific software. Here PDQUEST 2-D analysis Image analysis software was utilized for analysis of differential protein expressions. After 2DE step were completed, the protein spots in 2DE gel would be visualized with linear amount of detection fluorescence staining reagent, Sypro Ruby. And then 2DE images were acquisition with high performance scanner to obtain high resolution digitized 2DE image. Therefore, it could obtain good accuracy of investigation of differential protein expressions of *B. stearothermophilus* TLS33 in different stress conditions. Here, the scatter plots, generated by PDQUEST 2-D analysis software, were applied to be a tool for investigation of differential protein expression. However, there were other tools which PDQUEST 2-D analysis software aided analysis can be more accurate. Despite of computerized aid is able investigate differential protein expressions. In fact, 2DE method still has the limitation. This has been mentioned above. Therefore, it attempted to identify interesting proteins as many as possible. However, the obtained results still are very helpfully.

4.1.2.3 Protein synthesis and protein level profiles in response in each stress condition

As it has mentioned in above topics, not only one protein in the organisms affected from environmental changes but also other proteins in other pathway or regulon were controlled. On the other hand, many proteins probably were affected from alternation of their environment. Bioprocess could lead *B. stearothermophilus* TLS33 encountered the stress conditions. Salt, ethanol and lower temperature are case studies for investigation to global changes of protein complex in *B. stearothermophilus* TLS33. However, the study of completed genomic sequencing bacteria such as *B. subtilis* and *E. coli* seemed to be insufficient to investigate gram-positive thermophile, *B. stearothermophilus* TLS33.

4.1.2.3.1 Protein level profiles in salt stress

Changing of some selected proteins was demonstrated in figure 3.16 GroEL and GroES are proteins which involved in protein degradation and protein folding, GroEL were decreased in first 30 minutes and it was up-regulated in 60 minutes later. Tpx, detoxifying protein, was also appeared that in has been shifted to more acidic region in 2DE gel (Figure 3.16). So, it could be assumed that this protein was modified by ROS in cell resulting in formation of cysteine acid of –SH in cysteine residue molecule. Some of reports suggested that –SH of reactive cysteine will used for scavenger the exess ROS. So, O_2^- might oxidize –SH of cysteine and form –SOH, $-SO_2H$, $-SO_3H$. Therefore, it was postulated that the protein has more negative charge, resulting in shifting to more acidic region. This was demonstrated in figure 3.16. Mn-

SOD, functions to convert H_2O_2 to H_2O, was down-regulated. In addition, there was a report mentioned that oxidative stress also was resulted form salt stress as well (119). However, Mn-SOD is supposed to up-regulated because organisms have to eliminate H_2O_2, which is very harmful to the cell, by enzymatic mechanism. In this case, it might be conflict to the fact. The possible explanation for conflict might be that H_2O_2 acts as signaling molecule, therefore, elimination of H_2O_2 would not be necessary. L-lactate dehydrogenase (LDH), is an enzyme involved in glycolytic pathway. But it was demonstrated that this protein has been up-regulated as it was shown in figure 3.16. Eventhough LDH is an enzyme which functions as converting L-lactate to pyruvate but it also requires con-substrate $NADP^+$ and subsequently produce NADPH. NADPH can help detoxifying enzyme eliminate ROS and reduce the oxidized proteins return to their original form. For Universal stress protein, or Usp, currently, its function is still ambiguous. However, it has been found that this protein always up-regulated when gram-positive bacterium was under stress conditions. Likewise, EF-TU was reported that this protein probably was up-regulated during stress condition. In fact, this protein is very abundant protein, approximately 5 percentage of total of protein. And it protects amino-acyl-tRNAs from spontaneous hydrolysis as well as GTP-binding protein. In addition, this protein also is involved in protein synthesis. A literature suggested that salt stress condition induced EF-TU for protein synthesis and it might be an adaptation in this thermophile. Nevertheless, the down-regulation of PryB or aspartate Carbamoly transferase and the up-regulation of Fmt, Met-RNAi formyltransferase are still ambiguous because there was no any literatures strongly recommended that these proteins were changed due to salt stress. Actually, both of PyrB and Fmt are involved in amino acid and protein synthesis as well. Therefore,

further studying is necessary to ensure and other approach should be use to elucidate whether these proteins are necessary for adaptation of this thermophile to salt stress. In conclusion, salt stress seemed to effect on protein synthesis and glycolytic pathway. However, some proteins probably participating in salt stress response although they are low abundant protein, accordingly, the limitation of 2DE caused a number of proteins were missed.

4.1.2.3.2 Protein level profiles in ethanol stress

Similar to salt stress, ethanol stress induced group of detoxifying proteins and glycolytic pathway, for example, Tpx, ADH pyruvate dehydrogenase etc. The most of proteins induced by ethanol stress, are involved in glycolytic pathway such as pyruvate dehydrogenase, NADH dehydrogenase and acetaldehyde dehydrogenase. There was a literature has mentioned to *S. cerevisiae* expressed all these proteins participating on production of NADPH (120). NADPH is co-substrate that also participating in detoxifying mechanism because it serves as reducing agent. Thioredoxin and peroxiredoxin in redox signaling pathway and reducing mechanism of oxidized protein in oxidative stress of the organisms used NADPH for reducing themselves to original form. Compromisingly, PutA, is a protein requires $NADP^+$ and produces NADPH. It was up-regulated as well. Nevertheless, it has been found other proteins involved in other pathways such as HrcA, involved in transcription of class I heat shock heat shock gene (121), ABC ATPase, involved in protection against extreme temperature (115), and PPT, related in protection metabolism. Whereby, these proteins could not definite the relation of them as it has been suggested above.

These proteins are necessary to apply other method for ensure and conclude their importance.

4.1.2.3.3 Protein level profile in cold stress

In cold stress, markedly, CspB was up-regulated as well. In general CspB is the protein that is found in *B. subtilis* but not in *E. coli* (122). Therefore, CspB is a specific protein in gram positive bacteria. Also, peroxiredoxin were up-regulated in first 30 minutes. LysR is protein which serves as transcriptional regulator, while SrrB is protein which involves in signal transduction. This is corresponding to other stress conditions that the most of effected proteins relatively involved protein synthesis and oxidative stress. In conclusion, individual stress could induce protein that involved in oxidative stress and glycolytic pathway. Nevertheless, some proteins might be missed from surveying protein expression in this study. In addition, protein identification and bioinformatics, which have been available in internet, still can be insufficient to view the global protein expression. Therefore, it is supposed to apply non-gel based proteomics technique likewise isotope-coded affinity tags (ICAT) (123), iTRAQ™ for investigating the interesting missing proteins which they could not be visualized form gel-based techniques.

4.1.3) THE STUDYING OF POST-TRANSLATIONAL MODIFICATION OF AHPC OR PEROXIREDOXIN (PRX)

When *B. stearothermophilus* TLS33 was forced to stress conditions such 10% w/v NaCl salt stress, 10 % v/v ethanol stress and 25°C cold stress, it was found that individual stress could induce some of proteins. Among these proteins, there were number of enzymes that demonstrated that they are involved in redox-signaling pathway. One of those proteins is Peroxiredoxin (Prx) or AhpC. In general, Peroxiredoxin would be increased when the organisms are in the oxidative stress. Thus it seems to help to adaptation of the organisms. This protein not only is ROS scavenger but also redox signaling protein. Thus, it was attempted to investigate alternation of this protein by 2DE technique and LC-MS/MS technique.

4.1.3.1 Bacterial survival of *B. stearothermophilus* TLS33 under oxidative stress

H_2O_2 is weak oxidizing agents, they can react with cysteinyl-thiols in protein by formation of disulfide bonds. Figure 3.19 showed percentage of survival of this bacterium during it was encountered to the oxidative stress. Here, *B. stearothermophilus* TLS33 was treated with different concentrations of H_2O_2 (10, 50 100 and 500 µM). It has been found that at 10 µM of H_2O_2, this thermophile can resist to this concentration. For higher concentration of H_2O_2, it seemed that percentage of survival was begun to decrease at 50 µM of H_2O_2 which it is approximately 95-98%. And the higher concentration of H_2O_2 also caused percentage of survival was

decreased. Whereby, at 500 µM of H_2O_2, percentage of survival is approximately 85-88% within 120 minutes.

4.1.3.2 2DE analysis of *B. stearothermophilus* TLS33 and protein identification

From the changes of protein expression of this thermophile against to oxidative stress, interestingly four isoform spots located on low molecular weight approximately 27 kDa were obseverved. Individual spot form basic region to acidic region denoted as Prx I, Prx II, Prx III and Prx IV are different in p*I* with 5.0, 4.87, 4.81 and 4.79, respectively. This was calculated from Image Analysis software. Table 3.2 showed matched peptide sequences, p*I* and MOWSE score. In fact, 2 isoforms of Prx I and Prx II normally appeared in the cytosol of this bacterium. But in oxidative stress, it seems that this protein would shift to more acidic region corresponding to oxidative strength. This evidence could suggest according to previous studies. It could suggest that the reactive oxygen species (ROS) can oxidize Prx and modify it to become four isoforms when the bacterium encountered oxidative stress.

4.1.2.3 Acidic shift of Prx Isoform

Image Analysis software, Image master™ 2D platinum software was performed to generate 3-D viewings individual Prx isoform (Figure3.22A). Here, the image viewings showed Prx I normally located on basic region while Prx II, Prx III and Prx IV located on acidic region. In the presence of different of concentration of H_2O_2, The results in figure 3.22A and figure 3.22B could be suggested that at 10 µM

of H_2O_2 , Prx I still located on the basic form. Prx II appeared when bacterium was treated with 50 µM. In the mean time, Prx III appeared at this concentration as well. At 100 µM of H_2O_2, Prx II and Prx III seemed that they were increased. In addition, it was found that Prx IV appeared at 500 µM of H_2O_2. At this concentration, figure 3.24 showed that this protein would not be returned to its original form. Thus, we presume that the modified the cysteines with sulfenic acid in protein molecule may formulate the proteins to have positive charge and intend to shift the p*I* of protein. Therefore the level of oxidative stress directly affected to the modification of Prx isoform and the active-site cysteines may be modified by H_2O_2 to be obtain the acidic form (Figure 3.24). So far, the Prx II and Prx III and Prx IV abundances are corresponding to the increasing of H_2O_2. On other hand, alternation of Prx isoforms resulted form regulation of Prx. Otherwise, the previous studies have showed the evidences which indicated Prx was post-translational modified by H_2O_2.

4.1.3.4 Post-translation modification of AphC or Peroxiredoxin(Prx)

Here, According to hypothesis, the Prx isoforms would be modified with ROS and formed sulfinic acid (Cys-SO_2H). To characterize the modifications, LC- MS/MS were applied for investigating this modification. In this case, the sulfenic acid was hypothesized that it might be formed after the bacterium was treated with H_2O_2. Therefore, mass difference should be 135 mass units. Furthermore, it was found that some peptides which derived form tryptic digestion from Prx II and Prx III had mass different in peptide fragment mass. To confirm the hypothesis, these tryptic digestion peptides were analyzed by collision-induced dissociation. From MS/MS spectrum in

figure 3.25, it indicated the H_2O_2 oxidized –SH group in cysteine residue of Prx II and Prx III. And then a mass difference of 135 mass units was detected between b3 and b4 ion in 598 *m/z* of Prx III and Prx III and between b6 and b7 in 809 *m/z* of Prx III. According to the hypothesis, H_2O_2 would oxidized –SH group in another cysteine residue in Prx II and resulted in the protein shift to more acidic region and subsequently formed Prx II. Thus, it indicated the presence of 598 *m/z* and 809 *m/z* peak in Prx III where as was found that only 598 *m/z* is present in Prx II. For Prx IV, it was found that there was no any mass difference of 135 mass units in peptide sequence. But it suggested that this isoform might occur from overoxidization of –SH and formed $–SO_3H$ in stead of $–SO_2H$ (121,124). However, there are still more proteins that have similar behavior. In addition, there are many studies supported that ROS-oxidized proteins will shift to more acidic region.

4.1.4) USING CY3 MALEIMIDE FOR DETECTION OF THIOL OXIDIZED PROTEIN IN *BACILLUS STEAROTHERMOPHILUS* TLS33

4.1.4.1 Using Cy3 Maleimide to detect oxidative modified protein

In this study, thiol oxidized proteins were believed they are redox signaling pathway, were detected with Cy3 Maleimide labeling methodology. It was found that Cy3 Maleimide labeling oxidized proteins could visualize thiol oxidized proteins to be signaling proteins which are due to oxidized –SH such as disulfide bond and sulfinic acid can be utilized as H_2O_2 exchanger between individual oxidized proteins. In this study, Cy3 Maleimide was applied for detection of thiol oxidized protein. The

figure 3.28 demonstrated proteins which have been identified by MALDI-TOF. And the figure 3.29 demonstrated that Cy3 Maleimide labeling and staining with Sypro Ruby protein. It has been found that Sypro Ruby staining is able to visualize more protein spots rather than Cy3 Maleimide labeling methodology. However, the differential display of protein spots between control and treated sample could be viewed. This suggested that there still are some of proteins which were not modified by H_2O_2, so, they were not be labeled by Cy3 Maleimide. However, it was found that they were up-regulated in Sypro Ruby staining. Therefore, oxidative stress not only resulted in modification of –SH of the proteins but also induction of other protein expression. In this study, Pro Q Diamond was applied for investigating on oxidized proteins. Due to there was a literature mentioned that ProQ Diamond could visualize oxidized protein (122). In the figure 3.31A indicated protein in Cy3 Maleimide labeling, this protein was assumed that it had 3 isoforms while Cy3 Maleimide could visualize only 2 isoforms i.e. original form and more acidic form (from right to left side). This was observed in both of control and oxidative sample whereas this protein was observed all three isoforms. Cy3 Maleimide labeling method missed third isoform of this spot. According to figure 1.4 and report (121) it indicated $–SO_3H$ or sulfenic acid would be formed whereas it is overoxidized. Furthermore, there have been supported with a report (120-122) mentioned that this form would not be reduced back. Therefore, this protein isoform could not be labeled with Cy3 Maleimide methodology. While ProQ diamond could visualize only first isoform, In conclusion, Cy3 Maleimide method can indicate the oxidized proteins containing –SOH because –SOH is easily reduced back to –SH. Tpx protein was found that there are two isoform, acidic and basic form. From all protein visualization method, Cy3

Maleimide and Sypro Ruby could visualize all of two isoforms. Unlike, it seems that ProQ diamond could not visualize this protein. So, it suggested that this protein might have no specific group for ProQ Diamond staining, although it has been observed basic isoform of this protein. Furthermore, it was found this protein would be shift to more acidic region in 2DE gel which is same as Prx Protein, but both of Cy3 Maleimide methodology and Sypro Ruby could visualize both of its isoforms. For universal stress protein, this protein also had three isoforms. The figure 3.31C demonstrated that the visualization of this protein in individuals. In Cy3 Maleimide method, all of three isoforms of this protein were visualized. Unlike, in oxidative stress, it seemed that only two spots were visualized, furthermore, these spots were also split into two spots. And the first spot (right spot) was not visualized as well. Thus, this probably indicated that the first spot in control condition probably has –SOH within its molecule. In contrast, the first spot in oxidative stress was disappeared. So it suggested that, in oxidative stress, first spot might be form $–SO_2H$. On the other hand, the bacterium was under oxidative stress, -SOH group of universal stress protein probably be reduced by other proteins or enzymes which similar to Prx or Trx protein (126) In addition, in staining with Sypro Ruby, the first spot was also appeared. Therefore, this is supporting evidence that indicated first spot still located on the same place as control condition 2DE gel. Although it was split into two spots is due to oxidative conditions. For Pro Q Diamond staining of universal stress protein, only first two spots (from the right side) were visualized.

4.1.4.2 Protein identification and protein function classification

From Cy3 Maleimide labeling method, it could lead us view on thiol oxidized proteins which were believed that they might have potential to be redox-signaling proteins. Therefore, the proteins, were labeled with Cy3 Maleimide, were further identified with MALDI-TOF MS. These proteins have been shown in table 3.4. And then they were investigated their cysteine residues in their molecule, it was found that these proteins contain cysteine residues However, it still could not definite which cysteine residue is reactive. This should be investigated in further analysis. In addition, from image analysis, the protein expression ratio was investigated and it was shown in table 3.4 as well. Protein functions were obtained from protein database and they were attempted to classified into individual metabolisms such as detoxification, RNA synthesis or transcription regulation, Metabolism of carbohydrate and related compounds etc. In figure 3.32 showed the protein function classification histogram, it indicated that the major proteins, which could be visualized by Cy3 Maleimide method, are RNA synthesis, detoxification, Metabolism of amino acid and related of molecule including metabolism of carbohydrate and related compounds. Interestingly, Fructose-1,6-biphosphate aldolase (Fba), Glyceraldehyde phosphate dehydrogenase (GAPDH), L-lactate dehydrogenase (LDH) as well as Acetaldehyde dehydrogenase (ADH) were up-regulated with using Cy3 Maleimide labeling methodology, which these proteins involved in pentose phosphate pathway (Figure 3.33). These proteins seem to produce NADPH which was believed that it is a reducing agent in detoxifying mechanism (125). Except ADH, this enzyme seem to consume NADH, nevertheless, it probably could use acetaldehyde which was accumulated from the upstream pathway likewise 2-p-glycerate which obtained for GapDH step. In summary, Cy3

Maleimide method can be a method for detection of thiol oxidized proteins that also hypothesized that they are redox-signaling proteins.

4.1.5) TWO DIMENSIONAL DIFFERENTIAL IN-GEL ELECTROPHORESIS (2D-DIGE) OF THIOL OXIDIZED PROTEINS

DIGE is a new approach in comparative differential display proteomics. Previously this technology has hot been applied to direct analysis of thiol oxidized protein in *B. stearothermophilus* TLS 33. In this study, the DIGE technique has been successfully applied to identification thiol oxidized proteins from this bacterium. Proteomic analysis of thiol oxidized proteins in this thermophile employing 2D-DIGE technique highlights several advantages. First, since the two pools of protein extracts were separated in the same gel, the reproducibility compared to conventional 2D-gel separation is improved and the comparison of protein expression patterns is simplified. Second, the differences in protein expression between two populations of protein can be more accurately imaged, and differential protein expression easily identified, based on fluorescence of the labeled Cy3 and Cy5 dyes, thus providing accurate quantitation of protein changes. Finally, DIGE represents a step forward for high throughput analysis of 2DE-gels by its capability of automatic gel imaging, easy quantitation and comparison of gel images, and 50% fewer gels required for analysis (because of two pools of sample are separated in the same gel.) Moreover, according to the thiol oxidized proteins might be shift to acidic region in 2DE gel, this technique provides the feasibility to observe the differential proteins in 2DE gel by dual channel images i.e. the combination of red and green spots, consequently, those spots will

become yellow spots. Also, whenever, some spots appeared in either control or treated sample, those will markedly express in single color (green or red). For the investigation of thiol oxidized proteins in individual protein function, there have been the proteins related to redox metabolism seemed to shift to acidic region. This technique can provide more obvious observation of these proteins. However, the comparisons among protein visualization methods were discussed in table 4.1.

Table 4.1 The comparisons of the advantages and defects of individual protein visualization method

Method	Detection	Advantages	Defects
Sypro Ruby	General protein	Up/down regulation	Unable to detect any post-translational modification
ProQ Diamond	Phosphoproteins	1.Changes of phosphoproteins 2. Able to be post-labeling stained with Sypro Ruby	Unable to visualize different conditions in the same gel
Cy3 Maleimide	Thiol oxidized proteins	1.Changes of thiol oxidized proteins 2. Able to be post-labeling stained with Sypro Ruby	Unable to visualize different conditions in the same gel
DIGE	Thiol oxidized proteins	Able to visualize different conditions in the same gel	Unable to be post-labeling stained

4.2) CONCLUSION

Proteomics are science which focused on changes of protein within organisms cell. In which, this study applied several techniques in proteomic field such as two dimensional electrophoresis, MALDI-TOF, MALDI-TOF TOF, LC-MS/MS, image analysis, N-terminal sequencing including tryptic in-gel digestion. These techniques lead us to view changes of proteome level of *B. stearothermophilus* TLS33.

From this study, stress conditions can lead us to understand in adaptation network of this thermophile. CspB, GroEL, GroES, Prx and Tpx all are proteins which this bacterium has been produced within the cell. Therefore, these proteins might be applied in future work of this bacterium. In addition, cold shock stress in different temperatures can also indicate the proteins which are involving in signaling pathway in sporulation. Nevertheless, other proteins in signaling pathway of sporulation in this thermophile could not be observed, this might be caused in many factor such as limitation of 2DE, capacity of mass spectrometry even protein visualization techniques. However, at least, we could obtain knowledge from this study not slightly.

Prx protein was investigated its post-translational modification. Due to it was found that it had four spots in 2DE gel, same molecular weight but different in p*I*. Finally, from application of tandem mass spectrometry, LC-MS/MS, could indicate that this protein was modified by H_2O_2 and formed $–SO_2H$ or sulfenic acid of its cysteine residue. In which, there were report supported that this modification is a mechanism in signal transduction. In addition, Cy3 Maleimide labeling methodology was invented in purpose of detection of thiol oxidized proteins which are potential to

be redox-signaling proteins. Furthermore, these proteins were identified with MALDI-TOF MS and classified which are corresponding to their functions. It indicated that RNA synthesis and protein synthesis proteins are the most proteins which were affected from the oxidative stress. However, proteins participating in pentose phosphate pathway are also observed. Interestingly, these proteins are involved in production of NADPH which was believed that it is reducing agent in oxidative stress. 2D-DIGE is another novel technique for investigation of thiol oxidized proteins in this study. This technique can provide reproducibility of 2DE method as well as can assist to more obviously view on the differential display of the thiol oxidized proteins. In addition, the knowledge from this study is basic information in development of improvement utilization of this species, for instance, the genetic improvement for construction to resist to extreme condition such high concentration of salt or ethanol including unsuitable temperature of this species. Also, even investigation of stress proteins which might be different from model completed genome sequencing bacteria, such as *B. subtilis* or *E. coli* can be utilized in industrial work because of its thermostability.