Protein Produced by *Bacillus subtilis* ATCC11774 after Inducing with *Allium sativum* Extract

Hanina Mohd Noor, Hairul Shahril Muhamad, Salina Mat Radzi and Maryam Mohamed Rehan

***Abstract*—**Stressful environment especially in the presence of antimicrobial agents could increase the levels of protein produced by bacteria. Therefore, this study aims to explore the potential of natural antimicrobial compound, *Allium sativum* at low concentration in producing protein by *Bacillus subtilis* ATCC11774. The bacterial cells were exposed to 0.01 MIC of *A. sativum* in fermentation process at 30 ℃ and 37 ℃ for 12 h, 24 h, 48 h and 72 h. Analysis by Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE) showed that new intracellular proteins with approximate size of ~13 kDa and ~38 kDa, each was produced by *B. subtilis* after being treated with *A. Sativum* at 37 ℃ for 24 h and at 30 ℃ for 72 h respectively. Besides, new extracellular proteins approximately ~20 kDa and ~36 kDa in size, each was synthesized by *B. subtilis* prior to treatment with *A. sativum* at 37 ℃ for 48 h and 72 h subsequently. However, the proteins were expressed differently in which some were highly expressed, while some were repressed based on the intensity of protein bands appeared. Thus, protein production by *B. subtilis* ATCC11774 could be enhanced in the presence of 0.01MIC of *A. sativum*.

***Keywords*—**Protein, *Allium sativum*, *Bacillus subtilis* ATCC11774.

I. Introduction

BACTERIA exist in the environment are exposed to some variable factors, such as alterations in temperature, availability of nutrients and water, and the presence of toxic molecules that originate from their abiotic and biotic environment, causing the living conditions are far from the optimal. Therefore, bacteria need the capability of protecting DNA damages in order to adapt and survive in that stress condition. The bacterial response leads to transcriptional activation of genes whose products cope with a given physicochemical stress. Gene regulators respond to specific signals by stimulating or inhibiting transcription, translation or some other event in gene expression, so that the rate of protein synthesis is appropriately modified [1].

Antimicrobial compounds represent one of the many stresses that bacterial must sense and response to, in order to thrive in harsh environmental conditions [2]. Bacteria develop special defense mechanisms including the production of protein for survival. As a response to the stress such as antimicrobials, proteins are produced and the protein level is higher than the normal condition. The mechanism of increasing the protein levels occurs at transcriptional level [3]. The elevated expression of stress proteins is considered to be a universal response to adverse conditions, representing a potential mechanism of cellular defense against disease and a potential target for novel therapeutics [4].

Allium sativum has a broad antimicrobial activity against Gram-negative and Gram-positive bacteria, including the multidrug-resistant enterotoxicogenic strains, *Escherichia coli* [5]. However, the antimicrobial compounds could act as signaling molecules when used at low concentration. The antimicrobial agents at sub-minimal inhibitory concentration (sub-MIC) have the ability to cause global changes in gene transcription process by activating or repressing the specific genes of interest via the signal given concentration [6]. For example, a study indicated that sub-MICs of antibiotic can enhance and modulate the production of new phenazines, streptophenazines A-H with antimicrobial activity against *Bacillus subtilits* and *Staphylococcus lentus* in a marine *Streptomyces* isolate [7]. Besides, another research showed that the presence of *C. flexuosus* essential oil at 0.01 MIC could induced the production of Bacillopeptidase F by *B. subtilis* ATCC 21332 [8].

Naturally, bacteria could respond to any extracellular signals in the environment. However, there have been only few studies on the potential of natural antimicrobial compounds as a specific chemical signal that can trigger biological functions in bacteria. Therefore, this present study was carried out to determine the protein produced by *B. subtilis* ATCC117774 after inducing with low concentration of natural antimicrobial substance which is *A. sativum*.

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II. Materials And Methods

A. Allium sativum, Bacterial Strains and Culture Condition

Allium sativum was provided by Nilai local market outlets. *Bacillus subtilis* strain ATCC117774, obtained from American Type Culture Collection (ATCC) were grown in Mueller-Hinton Broth (Oxoid, USA).

B. Protein Production

Bacillus subtilis ATCC11774 cells were tested for their reactions to the presence of *A. sativum* extract. A bacterial colony was inoculated in 50 ml of MHB before being further agitated vigorously at 30°C and 37°C. *A. sativum* extract at concentration of 0.01 MIC was then added to each bacterial culture after 8 h of cultivation (which is log or exponential phase) and the culture was further agitated vigorously at 30°C and 37°C for 12 h to 72 h of fermentation period. A culture to which *A. sativum* extract was not added served as a control.

C. Protein Isolation

Extracellular proteins from cell-free supernatants and intracellular proteins from pellet were isolated according to method by [9]. After 12 h to 72 h of fermentation at 30ºC and 37ºC, each bacterial culture was centrifuged at 7000 × *g* for 6 minutes at 4ºC (Centrifuge, Eppendorf, USA) to separate the supernatant from pellet containing bacterial cells.

D. Extracellular Protein Extraction

Extracellular proteins from cell-free supernatants were extracted according to method by [9]. The supernatant containing extracellular proteins was collected and transferred to new tubes. About 80% (w/v) of ammonium sulphate (Sigma, St. Louis, Missouri, USA) was then added to the supernatant for protein precipitation process before being kept for 1 h at 4°C. The precipitated proteins were collected by centrifugation at 15000g for 20 min. The resulting pellet was resuspended in phosphate buffer solution (PBS, pH 6.8) before being further analyzed for protein identification by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein suspension was futher dialyzed at 4°C for 48 h and the dialyzed proteins were then mixed with Laemmli buffer (Bio-Rad, Singapore) in 1:1 ratio and heated at 95°C for 10 min before being loaded into a SDS-PAGE gel.

E. Intracellular Protein Extraction

Intracellular proteins from pellet were isolated according to method by [9]. The pellet cells which are the intracellular proteins were separated from fermentation medium and washed with phosphate-buffered saline (Cambrex, Bioscience, Belgium) twice, before being dissolved in sterile distilled water. The protein suspensions were then mixed with Laemmli buffer (Bio-Rad, USA) and β-mercaptoethanol (Sigma-Aldrich, USA) in 1:1 ratio before being heated at 95°C for 10 min and cooled on ice. The protein samples were ready to be further analyzed by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

F. Sodium Dodecyl Sulfate-Polyacylamide Gel Electrophoresis (SDS-PAGE)

Proteins were analyzed by electrophoresis on Any kD™ Mini-PROTEAN® TGX™ Precast Gel in a Protean III electrophoresis system (Bio-Rad, Hercules, CA) with Precision Plus Protein™ Dual Xtra Standards (Biorad, USA). The size of protein standard was from 2 kDa to 250 kDa.The protein bands made visible by staining with Biosafe coomasive blue (Bio-Rad, USA).

III. Results

The effects of *A. sativum* extract at concentration of 0.01 MIC on proteins production were studied and analysed by SDS-PAGE. As shown in Fig. 1, there is a new intracellular protein with approximate size of ~13 kDa was synthesized by *B. subtilis* ATCC11774 after being induced with *A. sativum* during 24 h of fermentation period at 37 °C. Besides, *B. subtilis* ATCC11774 could also produce a new intracellular protein approximately ~38 kDa in size after being treated with *A. sativum* at 30 °C for 72 h of incubation as shown in Fig. 2.

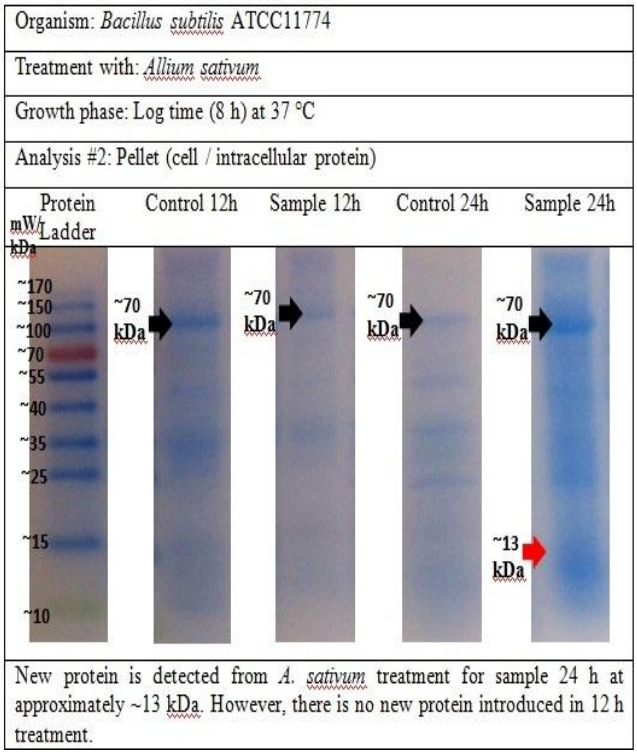

Fig.1 SDS-PAGE analysis on intracellular proteins produced by *B. subtilis* ATCC11774 in the presence of *A. sativum* at 37 °C for 12 h and 24 h of incubation: Lane (1) Protein Ladder (10 to170 kDa); Lane (2) in the absence of *A. sativum* after 12 h of fermentation period; Lane (3) in the presence of *A. sativum* after 12 h of fermentation period; Lane (4) in the absence of *A. sativum* after 24 h of fermentation period; Lane (5) in the presence of *A. sativum* after 24 h of fermentation period

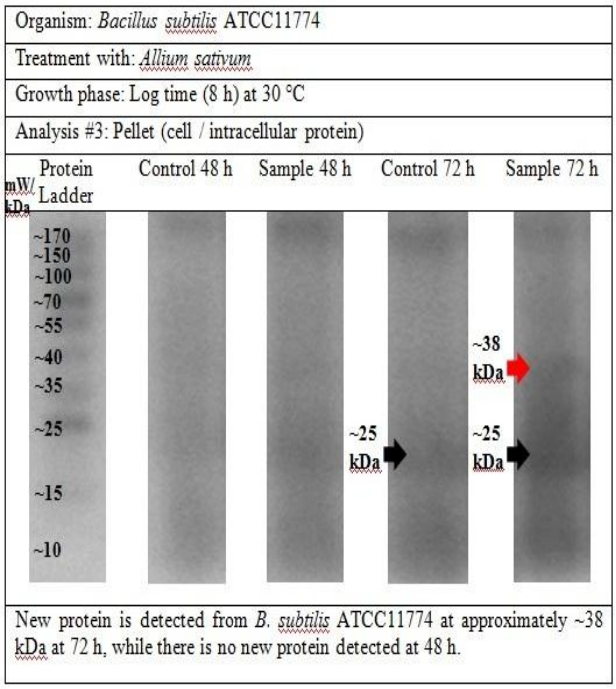

Fig.2 SDS-PAGE analysis on intracellular proteins produced by *B. subtilis* ATCC11774 in the presence of *A. sativum* at 30 °C for 48 h and 72 h of incubation: Lane (1) Protein Ladder (10 to 170 kDa); Lane (2) in the absence of *A. sativum* after 48 h of fermentation period; Lane (3) in the presence of *A. sativum* after 48 h of fermentation period; Lane (4) in the absence of *A. sativum* after 72 h of fermentation period; Lane (5) in the presence of *A. sativum* after 72 h of fermentation period

However, the intracellular proteins introduced by *B. subtilis* ATCC11774 prior to treatment with *A. sativum* as well as without the treatment of inducer were expressed differently. Some of the intracellular proteins were highly expressed and some of them were repressed based on the intensity of protein bands appeared. The intracellular protein approximately ~70 kDa in size which is synthesized by *B. subtilis* ATCC11774 after inducing with *A. sativum* at 37 °C for 12 h of incubation was repressed. Whilst, the similar intracellular protein with aproximate size of ~70 kDa produced by *B. subtilis* ATCC11774 with *A. sativum* treatment at 37 °C for 24 h was highly expressed (Fig. 1). Besides, high level of protein expression could also be observed for intracellular protein with approximate size of ~25 kDa produced by *B. subtilis* ATCC11774 prior to enhancing with *A. sativum* at 30 °C for 72 h of incubation (Fig. 2).

Extracellular proteins produced by *B. subtilis* ATCC11774 were also analyzed by SDS-PAGE. There are two new extracellular proteins introduced by *B. subtilis* ATCC11774 after inducing with *A. sativum* at 37 °C as shown on Fig. 3. The extracellular protein with approximate size of ~20 kDa was produced by *B. subtilis* ATCC11774 prior to enhancing with *A. sativum* at 37 °C for 48 h of incubation. *B. subtilis* ATCC11774 could also synthesized new extracellular protein approximately ~36 kDa after being treated with *A. sativum* and incubated at 37°C for 72 h.

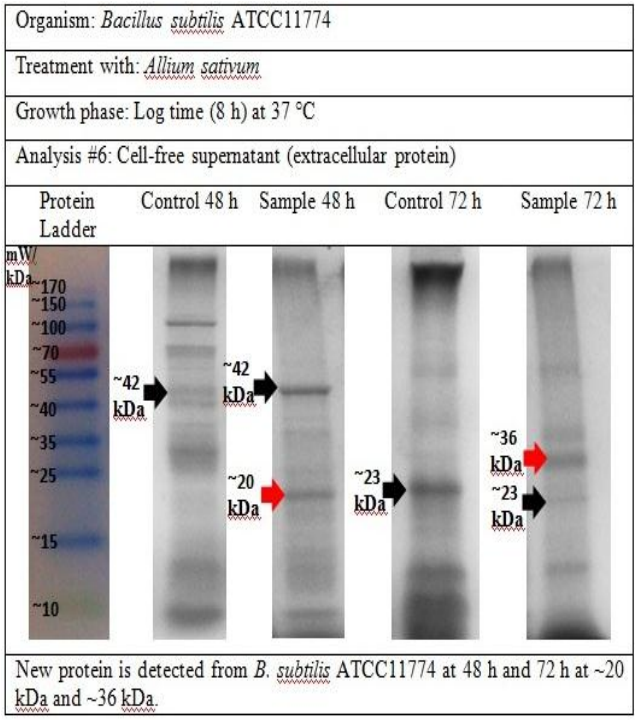

Fig.3 SDS-PAGE analysis on extracellular proteins produced by *B. subtilis* ATCC11774 in the presence of *A. sativum* at 37 °C for 48 h and 72 h of incubation: Lane (1) Protein Ladder (10 to 170 kDa); Lane (2) in the absence of *A. sativum* after 48 h of fermentation period; Lane (3) in the presence of *A. sativum* after 48 h of fermentation period; Lane (4) in the absence of *A. sativum* after 72 h of fermentation period; Lane (5) in the presence of *A. sativum* after 72 h of fermentation period

Besides, the extracellular proteins produced by *B. subtilis* ATCC11774 either with or without *A. sativum* treatments were expressed differently. Some of the extracellular proteins were highly expressed and some of them were repressed based on the intensity of proteins band appeared on SDS-PAGE gel. The ~42 kDa proteins was highly expressed by *B. subtilis* ATCC11774 prior to enhancing with *A. sativum* during fermentation process at 37 °C for 48 h. Whilst, the extracellular protein with approximate size of ~23 kDa synthesized by *B. subtilis* ATCC11774 was repressed after inducing with *A. sativum* and incubated at 37 °C for 72 h.

IV. Discussions

Bacteria often encounter drastic changes in their environment, including exposure to antimicrobial agents [10]. To ensure survival in facing these adversities, bacteria may adapt to changes in their immediate vicinity by responding to the imposed stress [11]. Bacteria have physiologic mechanisms to enabling them to survive and adapt in environments that can kill them. They produce endogenous and exogenous metabolites in order to protect DNA damages and regulate the expression of a variety of genes, which makes it able to adapt with different temperatures, pH, osmotic pressures and different concentration of nutrients and toxins [12]. For instance, previous study showed that in the presence of *C.*

flexuosus essential oil as an oxidative or osmotic stresses and in limited nutrients or starvation, *B. subtilis* ATCC21332 cells tend to produce new protein, identified as Bacillopeptidase F [8].

In this report, some effects of *A. sativum* extract on protein synthesis by *B. subtilis* ATCC117774 cells are described. When the bacterial cells were cultured for 12 h in the presence of 0.01 MIC *A. sativum* extract, there was no any new protein produced. The bacterial cells tend to produce new proteins when the incubation time was increased to 24h, 48 h and 72 h. It showed that *B. subtilis* ATCC21332 cells could maintain their normal physiological function within 12 h treatment with *A. sativum* extract. The bacterial cells were induced to introduce new protein in order to overcome the environmental stress caused by essential oil after 24 h to 72 h of incubation.

In this study, fermentation process was carried out for 12 h to 72 h before the microbial proteins were isolated and extracted to allow *B. subtilis* ATCC117774 to achieve its stationary phase. The microbial secondary metabolites are usually not produced during the phase of rapid growth (log phase). However, they synthesize secondary metabolites including microbial proteins during a subsequent production stage (stationary phase), which is when primary nutrient source is depleted [13]. Bacterial cells that have exhausted one or more essential nutrients experience fluctuations in the surrounding will enter the stationary phase of growth. At this stage, regulons that function in the production of proteins and enzymes are induced [14]. Hence, it is demonstrated that *B. subtilis* ATCC117774 were induced to produce new proteins or increase protein expression during stationary phase in order to adapt or survive in the presence of *A. sativum* extract.

The intracellular and extracellular proteins introduced by *B. subtilis* ATCC117774 prior to treatment with *A. sativum* were expressed differently. Some of the intracellular and extracellular proteins were highly expressed and some of them were repressed based on the intensity of protein bands appeared. It shows that the bacterial response toward the imposed stress is accomplished by changing the patterns of gene expression for those genes whose products are required to combat the deleterious nature of the stress. The up-regulation of the transcription of stress-responsive genes is achieved by the activation of transcription factors that interact with RNA polymerase to co-ordinate the gene expression [11].

There was a long-held belief that the Gram-positive soil bacteria, *B. subtilis* is a strict aerobe. But recent studies have shown that *B. subtilis* will grow anaerobically, either by using nitrate or nitrite as a terminal electron acceptor, or by fermentation [15]. With limited nutrients, bacteria do not continue their exponential growth indefinitely. Instead, they move into the stationary phase, cells lose viability and enter the death phase. In prolonged periods of nutrient depletion, a resistant subpopulation survives and the extended stationary phase ensues. To adapt in stress condition as such, alternative sigma factors enable bacterial RNA polymerase to transcribe an alternative of its genes. In the stationary phase, the starvation/stationary phase sigma factor is used to upregulate the expression of a number of genes [12].

One of the strongest and most noticeable responses of *B. subtilis* cells to a range of stress and starvation conditions is the dramatic induction of a large number of general stress proteins. The alternative sigma factor, sigma B is responsible for the induction of the genes encoding these general stress proteins that occurs following heat, ethanol, salt or acid stress, or during energy depletion. More than 150 general stress proteins/genes belong to this sigma B regulon, which is believed to provide the non-growing cell with a non-specific, multiple and preventive stress resistance. Sigma B-dependent stress proteins are involved in non-specific protection against oxidative stress and also protect cells against heat, acid, alkaline or osmotic stress [16]. Present study showed that in the presence of *A. sativum* extract, *B. subtilis* ATCC117774 cells tend to produce new intracellular and extracellular proteins. However, further analysis on protein sequencing should be done in future study.

V. Conclusion

B. subtilis ATCC117774 in the presence of *A. sativum* extract at low concentration were able to produce and secrete the intracellular and extracellular proteins. Therefore, further study can be done to identify, isolate and as well as to determine and evaluate the biological activities of these proteins.

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