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## **EFFECT OF ETHYL PENTANOATE ON ANTI- QUORUM ACTIVITY OF EXTRACELLULAR PROTEIN SECRETED BY *BACILLUS SUBTILIS* ATCC11774**

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### **Abstract:**

*Antimicrobials agents at low concentration could stress out bacteria by inducing proteins production via regulating the transcription and translation process. The interruption of bacterial quorum sensing (QS), or cell-to-cell communication is known to have the potential to weaken the bacterial pathogenicity by inhibiting their communication. This study aims to explore the potential of synthetic antimicrobial compound, ethyl pentanoate in stimulating proteins production by Bacillus subtilis ATCC11774 as well as to determine the anti-QS activity of microbial proteins produced. The bacterial cells were exposed to 0.01 MIC of ethyl pentanoate in fermentation process at 37°C for 48 h and 72 h respectively. The proteins produced were further isolated and analyzed by using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Results showed that a new extracellular protein with approximate size of 15 kDa was produced by B. subtilis ATCC11774 after being treated with ethyl pentanoate at 37°C for 48 h and 72h. Despites of new protein band production, there was a deletion of protein band with approximate size of 18 kDa on protein synthesized at 72 h of fermentation. Whilst, the anti-QS activity of microbial proteins produced by B. subtilis at 37°C for 48 h and 72 h was determined by agar- wells diffusion assay, resulting “halo” inhibition zone ranged from 10.00 ± 1.00 to 10.33 ± 0.56 in diameter. Therefore, B. subtilis ATCC11774 in the presence of ethyl pentanoate at 0.01 MIC could regulate the extracellular protein production and expression. The isolated protein also exhibited anti-QS activity.*

**Keywords:** Protein; Anti-Quorum Sensing (QS); Ethyl Pentanoate; Bacillus Subtilis ATCC11774.

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### **1. Introduction**

Bacteria may response to fluctuation of temperature, low concentration of nutrients available, oxygen stress of environment, cell envelope damage and other growth-compromising stresses, in which these unfavorable environmental conditions could stress out bacteria [1].

The bacteria will further undergo series of responses for them to survive in these stress environments. For example, as a stress response, the bacteria may synthesize antibiotics and extracellular enzymes, biofilm formation, and induction of resistance mutations. In spore-forming bacteria such as *B. subtilis*, spore formation is the most dramatic response to stress condition, in which the heat-resistant spores could help them to survive [1; 2].

Antimicrobials are one of the stresses that could affect the bacterial growth. However, the bacteria may respond to the antimicrobials by producing specific proteins such as bacteriocins for its survival. In stress condition, the protein level produced was higher than in the normal condition. Antimicrobials agent at sub-minimal inhibitory concentration (sub-MIC) or low concentrations able to affect the gene transcription process by activating or repressing gene transcription and expression [3].

A flavor ester or ethyl pentanoate is bioactive compound, in which it can inhibit the bacterial growth at high concentration. However, at low concentration it could act as signaling molecules [3]. Ethyl pentanoate exhibited antimicrobial activity towards several strains of bacteria including *B. subtilis*, *Escherichia coli*, *Salmonella typhimurium* and *Staphylococcus aureus* [4]. The inhibitory action of ethyl pentanoate is unrelated to pH but closely related to their lipid solubility and it likely to act on the cell membranes [5] Thus, attempt was made to use flavor ester or ethyl pentanoate as a bacterial stress inducer.

Quorum sensing (QS) is familiar as bacterial intercellular communication that regulates the pathogenesis of many medically vital organisms [6]. The QS allow bacteria to observe the surroundings for other bacteria as well as to adjust the behavior on a population-wide scale, in response to changes in the number of species exist in a community [7]. Therefore, the compounds with anti-quorum sensing (anti-QS) activity have potential to attenuate the bacterial pathogenicity by inhibiting the cell-to-cell communication [8; 9].

Naturally, bacteria could respond to any extracellular signals in environment. However, there is still a limited study on synthetic antimicrobial compound in which could act as signaling molecules at low concentration to activate the biological function in bacteria. Besides, there is few studies on anti-QS of proteins or peptides isolated from bacteria. Therefore, this present study was carried out to explore the potential of synthetic antimicrobial compound, ethyl pentanoate in stimulating proteins production as well as to determine the anti-QS activity of extracellular proteins secreted by *Bacillus subtilis* ATCC11774.

## 2. Materials and Methods

### 2.1. Ethyl Pentanoate, Bacterial Strains and Culture Condition

Ethyl pentanoate was purchased from MERCK-Schuchardt. Two bacterial strains used, including *Bacillus subtilis* ATCC11774 and *Chromobacterium violaceum* ATCC12472, were obtained from American Type Culture Collection (ATCC). *B. subtilis* ATCC11774 was grown in Mueller-Hinton Broth (Oxoid, USA) before being further used to study the bacterial stress inducer. Whilst, *C. violaceum* ATCC12472 was grown in Nutrient Broth (Merck, Germany) before being further streaked on Luria Bertani agar (Sigma-Aldrich, USA) and used for anti-quorum sensing assay.

## 2.2. Microbial Protein Production

Fermentation process for microbial proteins production was conducted based on method by Lash *et al.* (2005) [10]. In this process, a colony of *B. subtilis* ATCC11774 was inoculated in conical flask containing 50 ml of MHB before being further shaken vigorously at 37°C. Ethyl pentanoate at concentration of 0.01 MIC in which used as stress inducer, was then added into the fermented broth during log phase of bacterial growth, which is after 8h of cultivation. A culture to which ethyl pentanoate was not added could serve as a control. Both bacterial cultures with or without treatment with ethyl pentanoate were further shaken vigorously at 37°C for 48 h and 72 h of fermentation period respectively.

## 2.3. Microbial Protein Isolation

Extracellular proteins from supernatants and intracellular proteins from pellet were isolated based on method by Lash *et al.* (2005) [10]. After 48 h and 72 h of fermentation, each bacterial culture was centrifuged at  $7000 \times g$  for 6 minutes at 4°C (Centrifuge, Eppendorf, USA) to separate the supernatant from pellet containing bacterial.

## 2.4. Extracellular Protein Extraction

Extracellular proteins from supernatant were extracted based on method by Lash *et al.* (2005) [10]. The supernatant containing extracellular proteins was collected and transferred to new tubes. The supernatant was further added with 80% (w/v) of ammonium sulfate (Sigma, St. Louis, Missouri, USA) and incubated for 1 h at 4°C for protein precipitation. The precipitated proteins were then collected by centrifugation at  $15,000 \times g$  for 20 minutes at 4°C. The resulting pellet was then dissolved in phosphate-buffered solution (PBS, pH 6.8) before being analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for protein identification. The proteins suspension was further dialyzed at 4°C for 24 h. The dialyzed proteins were then mixed with Laemmli buffer (Bio-Rad, USA) in 1:1 ratio and heated at 95°C for 10 min before being loaded into SDS-PAGE gel.

## 2.5. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The isolated extracellular proteins were separated and identified using SDS-PAGE (Bio-Rad, USA) based on method by Laemmli (1970) [11]. The protein samples were analyzed via electrophoresis on Any kD™ Mini-PROTEAN® TGX™ Precast Gel in a Protean III electrophoresis system (Bio-Rad, Hercules, CA) with Thermo Scientific PageRulerPrestained Protein Ladder (Bio-rad, California, USA). The size of protein standard used as molecular ruler ranging from 10 to 170 kDa. The protein bands made visible by staining with Biosafe coomassive blue (Bio-Rad, USA).

## 2.6. Screening of Anti-Quorum Sensing of Extracellular Proteins via Agar-Wells Diffusion Test

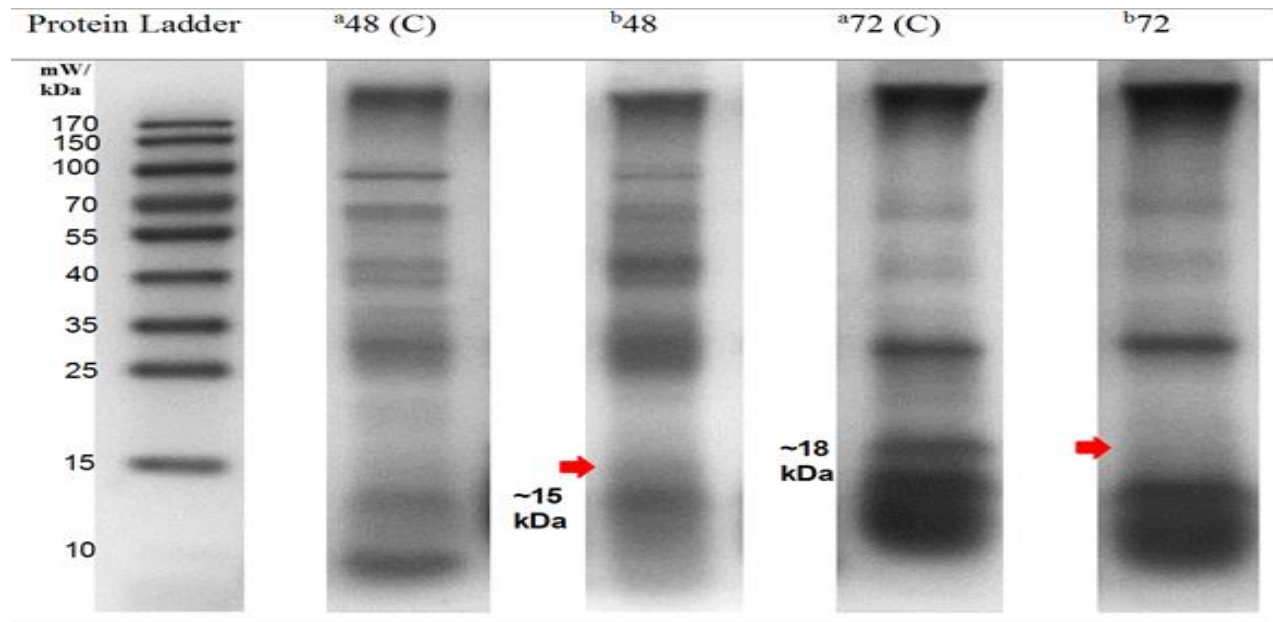
Screening on anti-quorum sensing of microbial proteins secreted by *B. subtilis* ATCC11774 was done based on method by Adonizio *et al.* (2006) [12] and Yeo and Tham (2012) [13]. In this anti-

QS assay, 5 ml of molten Luria Bertani Agar (Sigma-Aldrich, USA) were seeded with 100 µl of an overnight LB culture of *C. violaceum* ATCC12472. It was gently mixed before being further poured over the surface of a solidified Luria Bertani Agar plate as an overlay. Wells with 5 mm in diameter were made on the solidified agar using a sterile borer. A 10 µl of each isolated extracellular protein samples with different parameters of fermentation process was loaded into respective wells before being further incubated at 37°C and the diameter of clear inhibition zone or halo zone were measured after 24 h of incubation.

### 3. Results and Discussion

*B. subtilis* ATCC11774 was grown in the presence of ethyl pentanoate as stress inducer. The effect of ethyl pentanoate at concentration of 0.01 MIC on protein production were studied and analyzed by SDS-PAGE. As shown in Fig. 1, there is a new extracellular protein with approximate size of 15 kDa was synthesized by *B. subtilis* ATCC11774 after being induced with ethyl pentanoate during 48 h and 72 h of fermentation period at 37°C. Besides, the extracellular protein produced by *B. subtilis* ATCC11774 either with or without ethyl pentanoate 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 observed using SDS-PAGE analysis. Despite of new protein band production, there was a deletion of protein band with approximate size of 18 kDa on protein synthesized at 72 h of fermentation.

Anti-quorum sensing activity of extracellular protein secreted by *B. subtilis* ATCC11774 in the presence of ethyl pentanoate was determined by agar-wells diffusion test. As a control, protein produced without prior treatment with ethyl pentanoate was also used to study the anti-quorum sensing activity. The isolated proteins after each treatment with 0.01 MIC of ethyl pentanoate and incubated for 48 h or 72 h were labelled as 48 h and 72 h respectively. Whilst, the control protein samples that produced without treatment with ethyl pentanoate either incubated for 48 h or 72 h were labelled as 48 h (C) and 72 h (C) subsequently. The spectrum of anti-QS activity of proteins against *C. violaceum* ATCC12472 was determined based on the size of inhibition zone or “halo” inhibition zone formed as shown in Fig.2. Generally, the extracellular protein produced by *B. subtilis* ATCC11774 at 37°C for 48 and 72 h exhibited anti-QS activity with “halo” inhibition zone ranged from  $10.00 \pm 1.00$  to  $10.33 \pm 0.56$  in diameter as summarized in Table 1. Besides, protein samples without prior treatment with ethyl pentanoate also indicated anti-quorum sensing activity.



A new protein with approximate size of 15 kDa was detected for proteins sample produced at 37°C within 48 h of incubation and deletion of protein band with approximate size of 18 kDa for protein sample produced within 72 h of fermentation.

Figure 1: SDS-PAGE analysis on extracellular proteins produced by *B. subtilis* ATCC11774 in the presence of ethyl pentanoate at 37°C for 48 h and 72 h

Note: Lane (1) Protein Ladder (10 -170 kDa); Lane (2) Extracellular protein produced in the absence of ethyl pentanoate after 48 h of fermentation period; Lane (3) Extracellular protein in the presence of ethyl pentanoate after 48 h of fermentation period; Lane (4) Extracellular protein in the absence of ethyl pentanoate after 72 h of fermentation period; Lane (5) Extracellular protein in the presence of ethyl pentanoate after 72 h of fermentation period.

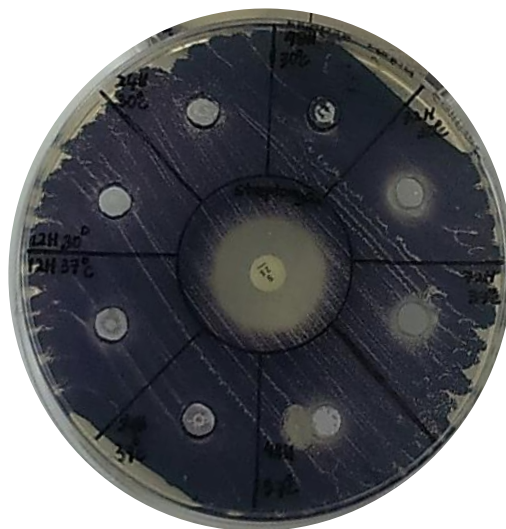


Figure 2: The Formation of QS inhibition zone by agar-wells diffusion Test



Note: Formation of clear halo zone which represent existence of anti-Quorum Sensing activity of the test samples. Streptomycin Sulphate was supplied at the center of the agar plates as a positive control.

Table 1: Spectrum of anti-Quorum Sensing Activity of Protein Produce by *B. subtilis* ATCC11774 at 37°C via Agar-Wells Diffusion Assay

Test/ Control Samples	Diameter of QS inhibition (mm)	Anti-QS Activity
<sup>a</sup> 48 h	<sup>c</sup> 10.33 ± 0.56	<sup>c</sup> +
<sup>a</sup> 72 h	<sup>c</sup> 10.00 ± 1.00	<sup>c</sup> +
<sup>b</sup> 48 h (C)	<sup>d</sup> 7.67 ± 0.56	<sup>c</sup> +
<sup>b</sup> 72 h (C)	<sup>d</sup> 8.33 ± 1.12	<sup>c</sup> +
<sup>e</sup> MHB + E. P	<sup>d</sup> 6.00 ± 0.00	<sup>d</sup> -
<sup>f</sup> MHB	<sup>d</sup> 6.00 ± 0.00	<sup>d</sup> -
<sup>g</sup> S	<sup>c</sup> 20.00 ± 0.00	<sup>c</sup> +

<sup>a</sup>48 h and 72 h: Protein isolated after being induced with 0.01 MIC of ethyl pentanoate; <sup>b</sup>48 h (C) and 72 h (C): Protein isolated without being induced with 0.01 MIC of ethyl pentanoate; <sup>c</sup>+ Has zone of inhibition = Has anti-Quorum Sensing activity; <sup>d</sup>- No zone of inhibition = No anti-Quorum Sensing activity; <sup>e</sup>MHB + E. P: Mueller Hinton Broth with 0.01 MIC of ethyl pentanoate; <sup>f</sup>MHB: Mueller Hinton Broth; <sup>g</sup>S: Streptomycin sulphate as positive control Spectrum of anti-quorum sensing activity: <sup>c</sup>+ Diameter of inhibition zone ≥ 7.83 mm = Has anti-Quorum Sensing activity; <sup>d</sup>- Diameter of inhibition zone ≤ 6.00 mm = No anti-Quorum Sensing activity.

### 3.1. Effect of Ethyl Pentanoate as Protein Inducer

An unfavorable environmental condition including fluctuation of temperature low concentration of nutrient available, oxygen stress of environment as well as exposure to antimicrobial agents could stress out bacteria [1]. In order to ensure the survival in facing these adversities, bacteria may adapt to changes in their immediate vicinity by responding to the imposed stress [14]. Bacteria has physiologic mechanisms to enable them to survive and adapt in environment that can kill them. They produce endogenous and exogenous metabolites to protect DNA damages and regulate the expression of a variety of genes, which makes it able to adapt with different concentration of nutrients and toxins [15].

In this report, *B. subtilis* ATCC11774 cells tend to produce a new protein with approximate size of 15 kDa when incubated at 37° for 48 h and 72 h. It showed that *B. subtilis* ATCC11774 cells could maintain its normal physiological function in earlier treatment with ethyl pentanoate. However, the bacterial cells were induced to introduce or secrete new protein during stationary phase which is after 48 h of incubation for them to adapt with the environmental stress caused by ethyl pentanoate. The microbial proteins or bacteriocins is grouped into four classes: Class I bacteriocins are modified lantibiotics that are less than 4 kDa, while Class II bacteriocins are small, heat stable, non-modified peptides and less than 10 kDa. Members of Class III are large, heat-labile proteins and larger than 30 kDa, while Class IV bacteriocins consists of cyclic peptides [16]. Based on this classification of bacteriocins' size, the new protein produced in this study might be categorized under Class II bacteriocins (15 kDa), which is a small and heat stable peptides. However, further analysis on protein sequencing should be done for further study.

### 3.2. Production of Extracellular Protein

In this study, the bacterial cultures were fermented at temperature of 37°C for two distinctive of time, including 48 and 72 h respectively, before being proceeded with protein isolation and extraction. During a subsequent production stage, in which the stationary phase where the primary nutrients source is depleted, the microbial secondary metabolites are synthesized. The bacterial cells that have used up the essential nutrients will expose to changes in its surrounding and enter the stationary phase of growth. Then, the regulons that function in the production of extracellular proteins and enzymes are induced [17]. Therefore, *B. subtilis* ATCC11774 cells were induced to produce new protein or increase protein expression during stationary phase due to adaptation changes in its surrounding caused by ethyl pentanoate.

The extracellular proteins introduced by *B. subtilis* ATCC11774 prior treatment with ethyl pentanoate were expressed differently. Some of the extracellular protein were highly expressed and some of them were repressed based on the intensity of protein bands appeared. Despite of new protein band production, there was a deletion of protein band with approximate size of 18 kDa on protein synthesized at 72 h of fermentation. It showed that the response to the enforce stress is achieved by changing the patterns of gene expression to combat the deleterious nature of the stress [18]. The up-regulation of the transcription of stress responsive genes is accomplished by activating the transcription factors that interact with RNA polymerase to co-ordinate gene expression [14].

### 3.3. Effect of Extracellular Protein Produced on Antimicrobial Activity Test

Agar-wells diffusion method is a primarily qualitative test that can be used as preliminary screening of anti-QS. The anti-QS activity or QS inhibition is based on the ability of certain test sample in inhibiting the growth of *C. violaceum*. It showed by the formation of 'halo' zones of inhibition or loss of purple pigment surrounding the well [19]. In this study, the extracellular protein produced by *B. subtilis* ATCC11774 at 37°C for 48 and 72 h exhibited anti-QS activity. It showed that *B. subtilis* ATCC11774 could produce bioactive protein prior to enhancing with ethyl pentanoate. However, the control protein samples without inducing with ethyl pentanoate also showed anti-QS activity. *Bacillus* sp. is known as producer of a broad spectrum of bioactive peptides that has a great potential in biotechnological and biopharmaceutical application [20]. Besides, *Bacillus* sp. could produce lipopeptides which acts as biosurfactants and peptide antibiotics with potent inhibition activity towards other bacteria [21]. Therefore, *B. subtilis* itself has an ability in producing bioactive protein that has anti-QS activity towards *C. violaceum*.

The anti-pathogenic or anti-QS compounds, in contrast to antibacterial compounds, neither kill bacteria nor stop their growth and are not related to the development of resistant strains. Instead, these compounds attenuate the expression of the genes responsible for pathogenesis by interfering with bacterial communication system like the quorum sensing activities [22]. The extracellular proteins secreted by *B. subtilis* ATCC11774 after being treated with ethyl pentanoate showed certain ranges of anti-QS activity based on the size of 'halo' inhibition zone formed. However, the size of inhibition zones might be influenced by volatilization of antimicrobial substances, wells size, amount of compound added to well, adsorption by the well, type of agar, agar strength, pH, volume of agar, and microbial strains used [19].

### 3.4. Adaptation of *B. Subtilis* towards Stresses

In recent years, *B. subtilis* has been shown to have capability to be a facultative bacterium in which, capable of growing with nitrate or nitrite as the electron acceptor or growing by fermentation in the absence of oxygen [23]. Bacteria do not continue their exponential growth indefinitely with limited nutrients. Instead, they move into stationary phase, where cells lose viability and enter death phase [15]. Consequently, they have developed sophisticated responses in which modulated by the remodeling of protein complexes and by phosphorylation-dependent signal transduction systems, to adapt and survive towards a variety of insults. These signaling systems affect transcriptional regulons either by activating an alternative sigma factor subunit of RNA polymerase such as sigma B in *B. subtilis* [14].

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 large number of general stress proteins. The alternative sigma factor, sigma B is responsible for the induction of genes encoding these general stress proteins that occurs due to heat, ethanol, salt or acid stress, or during depletion of energy. More than 150 general stress proteins/genes belong to this sigma B regulon that believed to provide 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 as well as protect cells against heat, acid, alkaline or osmotic stress [24]. This present study showed that *B. subtilis* ATCC11774 tend to produce new extracellular protein with anti-QS activity in the presence of ethyl pentanoate. However further analysis on protein sequencing should be done in future study.

### 4. Conclusion

*Bacillus subtilis* ATCC11774 in the presence of ethyl pentanoate at low concentration could produce or secrete the extracellular protein with anti-quorum (QS) sensing activity.

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