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EFFECTS OF *Cymbopogonflexuosus* ESSENTIAL OIL AND PH ON ANTI-COAGULANT ACTIVITY OF EXTRACELLULAR PROTEINS SECRETED BY *Bacillus subtilis* ATCC21332

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Abstract: Blood clotting is an emerging problem that seriously threatens the health of human beings such as strokes and heart attacks. For the treatment, anti-coagulants are used in which it could prevent and interrupt the process involved in the formation of blood clots. Microbial proteases have now been attracted since it is cost effective and no side effects than typical anti-coagulant agents. The protease enzymes were discovered from many microorganisms including *Bacillus* sp. Previous study reported that *B. subtilis* could produce a serine protease, known as Bacillopeptidase F with anti-coagulant activity. However, study on anti-coagulant activity of microbial protein secreted by *B. subtilis* after being induced with *Cymbopogonflexuosus* essential oil and cultured at difference pH media is still limited. In this present study, *B. subtilis* ATCC21332 cells were treated with a low concentration (0.01 MIC) of *C. flexuosus* essential oil and cultured in difference pH media (pH 6, pH 7 and pH 8) at 30°C for 72 h of fermentation. The extracellular proteins were then extracted and precipitated by using 80% of ammonium sulfate before being further identified using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis(SDS-PAGE) and tested for anti-coagulant activity. SDS-PAGE analysis exhibited a proteins profile with a band in approximate size of 30 kDa was appeared for the treated bacteria with *C. flexuosus* essential oil and cultivated at three difference pH of media. The extracellular proteins secreted by *B. subtilis* ATCC21332 after being treated with 0.01 MIC of *C. flexuosus* essential oil and cultivated either at pH 6 or pH 7 or pH 8 could also prevent blood clotting. However, the extracellular proteins produced by *B. subtilis* ATCC21332 without inducing with 0.01 MIC of *C. flexuosus* essential oil only exhibited anti-coagulant activity when the bacterial cells were cultured at pH 7. As a conclusion, *B. subtilis* ATCC21332 can be enhanced to produce anti-coagulant enzymes after being treated with low concentration of *C. flexuosus* essential oil and cultivated in natural pH media or even in slightly basic or slightly acidic environment. Further study should be done to purify, identify and analyze the anti-coagulant enzymes from *B. subtilis* ATCC21332.

Keywords: *Cymbopogonflexuosus* Essential Oil, pH effect, Anti-Coagulants Activity, Extracellular Proteins, *Bacillus subtilis* ATCC21332.

INTRODUCTION

Cardiovascular diseases are primary causes of death throughout the world [1]. The fundamental problem is the adhesion of fibrin in the blood vessels that leads to thrombolysis

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or fibrinolysis. Many thrombolysis agents were used for the treatment. However, the microbial fibrinolytic enzymes have now attracted since it is cost effective and no side effects than typical thrombolytic agents. The enzymes were discovered from many microorganisms, including *Bacillus sp.* isolated from fermented foods [2]. Bacillopeptidase F is one of the main microbial fibrinolytic enzymes which might play an important role in preventing the thrombotic disease [3].

Bacillus sp.produce a broad spectrum of bioactive peptides with great potential for biotechnological and biopharmaceutical applications. A well-known class of such compounds includes the lipopeptides surfactin, fengycin, iturin, mycosubtilins, and bacillomycins which are amphiphilic membrane-active biosurfactants and peptide antibiotics with potent antimicrobial activities [4-6]. Besides, this sporeforming soil bacterium also produces and secretes protease and other types of exoenzymes at the end of its exponential phase of growth The major extracellular proteolytic enzymes include the neutral protease, subtilisin or alkaline. It has long been known that *B. subtilis* could produce an extracellular serine protease with a high esterolytic activity [7].

Bacteria often encounter drastic changes in their environment, including fluctuations in the level of external oxygen and starvation. In order to adapt and survive in these environments, bacteria need the capability of protecting DNA damages by endogenous and exogenous metabolites and regulating the expression of a variety of genes, which makes it able to adapt to different temperatures, pH and osmotic pressures, as well as oxidative and ultraviolet light stresses [8; 9].

Antimicrobials represent one of the many stresses that a microbial pathogen must sense and response to, in order to thrive in harsh environmental conditions that allow the cell to cope with drug-induced stress. Such mechanisms include metabolic alterations that minimizing the toxicity of the drug, as well as the activation of chaperones and signal transduction cascade dedicated for sensing and responding to various stress [10]. Study showed that the proteins levels are increased for the bacteria to survive in stressful surroundings, such as in the presence of antibiotics [11].

Antibiotics are bioactive compounds that can serve as weapon in microbial communities at high concentrations due to their inhibitory activity toward other microorganisms. In ecological environments, these compounds may be at lower concentrations and likely play additional roles as signalling molecules [12]. Antimicrobial agents or antibiotics with different structures and modes of action at sub-minimal inhibitory concentrations (sub-MICs)

have the ability to cause global changes in gene transcription [13]. It appears that many antimicrobial agents or antibiotics, when used at low concentrations, have in common the ability to activate or repress gene transcription, which is distinct from their inhibitory effect [14]. For example, sub-MICs of antibiotics were found to enhance and modulate the production of new phenazines, streptophenazines A-H, in a marine *Streptomyces* isolate [15]. Previous study showed that *Bacillus subtilis* ATCC21332 in stressful condition with the presence of *C. flexuosus* essential oil at low concentration (0.01 MIC) could induce the production of bioactive protein recognized as Bacillopeptidase F [16]. The Bacillopeptidase F has the ability to degrade fibrin in blood clot and some reports also mention its function in anticoagulant of bloods [3]. However, study on anti-coagulant activity of microbial protein produced by *B. subtilis* after being induced with *C.flexuosus* essential oil and cultured at difference pH media is still limited. Therefore, this present study will focus on the roles of antimicrobial compounds (*Cymbopogonflexuosus* essential oil)in regulating anticoagulant proteins production by bacteria, which is *Bacillus subtilis* ATCC21332 after being cultured in difference pH media.

MATERIALS AND METHODS

A. Essential Oils, Bacterial Strains and Culture Conditions

Essential oil extracted from *C. flexuosus* was provided by Al-muqarram Holdings Sdn Bhd.*Bacillus subtilis* strain ATCC21332, obtained from American Type Culture Collection (ATCC) were grown in Mueller-Hinton Broth (MHB; Oxoid, USA).

B. Protein Production by Fermentation Process

Fermentation process was done according to method by [16]. In this fermentation process, *Bacillus subtilis* ATCC21332 cells were cultured in the presence of essential oil with different pH values. A single colony of *B. subtilis* ATCC 21332 was inoculated in 20ml of MHB media and incubated at 30°C for 24h. A 10µl of this an overnight culture was thentransferred into each 50ml of MHB media with different pH values (pH 6, 7 and 8) respectively before being further agitated vigorously at 30°C. After each of the bacterial culture reached the log phase of growth which is 8 h of cultivation, 0.01 MIC of *C. flexuosus* essential oil was then added before being further shaken vigorously at 30°C for 72 h. A culture to which essential oil was not added and using MHB media without changing the pH served as a control. The experiment was performed in duplicate.

C. Protein Extraction

The secreted extracellular proteins were extracted based on method by [17]. The bacterial cells were separated from the suspension by centrifugation at 12000 x gfor 15 min at 4ºC. The supernatant was collected and filtered with 0.2µm Minisart Sterile Filter before being transferred to a new tube.A80% (w/v) of ammonium sulphate was then added to the filtered supernatant for protein precipitation process before being kept for 1 h at 4^{o}C.The precipitated proteins were collected by centrifugation at 15000 x g for 20 min. The resulting pellet was dissolved in phosphate buffer solution (PBS, pH 7.3) and dialyzed or desalted using dialysis tubing (Sigma-Aldrich, USA) with 12400 Da cut-off for 24 h at 4°C, before being further evaluated onanticoagulant activity and protein analysis using sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel (SDS-PAGE).

D. Protein Identification using 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 dialyzed proteins were mixed with Laemmli buffer (Bio-Rad, Singapore) in 1:1 ratio and heated at 95°C for 5 min before being loaded into SDS-PAGE gel and electrophorized at 80 V. The sizes of protein standard used are from 2kDa to 250kDa. The protein bands made visible by staining with Biosafe coomasive blue (Bio-Rad, USA).

E. Anticoagulant Activity of Extracellular Protein on Human Blood

Anticoagulant of extracellular protein secreted by *Bacillus subtilis* ATCC21332 was done based on method by [18]. Each of the dialyzed extracellular proteins samples was added with fresh human blood samples. The fresh human blood samples added with 1 mL Tris–HCl served as a control. After being gently mixed, each of the mixtures was then incubated at 37°C for 30 min. The anticoagulant activity or effect of each dialyzed extracellular proteins samples on human blood was then observed.

RESULTS

B. subtilis ATCC21332wascultured in stress environment which is in the presence *C. flexuosus* essential oil with three different pH media. The effects of the essential oil at concentration of 0.01 MIC and three different pH media on protein production with anti-coagulant were studied. SDS-PAGE analysis exhibited a proteins profile with a band with approximate size of 30 kDa was appeared for the treated bacteria with *C. flexuosus* essential oil and cultivated at three difference pH of media as shown in Fig. 1. The result also showed that the 30 kDa extracellular proteins were highly expressed when the bacterial cells were

cultured in three difference pH media with the presence of *C. flexuosus* essential oil. This was based on the intensity of protein bands observed in SDS-PAGE analysis. In anticoagulant activity assay, the extracellular proteins secreted by *B. subtilis* ATCC21332 after being treated with 0.01 MIC of *C. flexuosus* essential oil and cultivated either at pH 6 or pH 7 or pH 8 could prevent blood from clotting as shown in Fig. 2 Fig. 3 and Fig. 4 subsequently. However, the extracellular proteins produced by *B. subtilis* ATCC21332 without inducing with 0.01 MIC of *C. flexuosus* essential oil only exhibited anti-coagulant activity when the bacterial cells were cultured at pH 7.

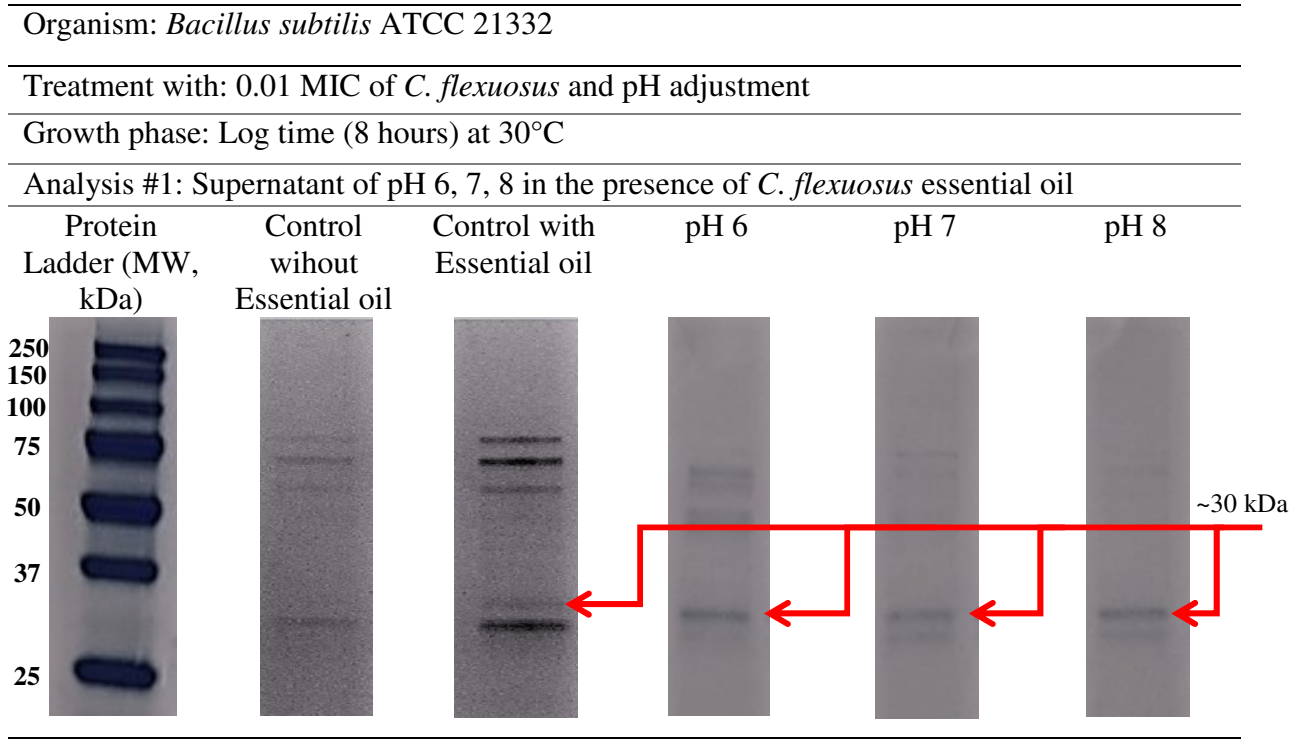

Fig. 1. SDS-PAGE analysis on extracellular proteins produced by *B. subtilis* ATCC21332 in the presence of *C. flexuosus* essential oil with media pH adjustment at 30°C for 72 h of fermentation

Note: Lane (1) Protein Ladder (25-250 kDa); Lane (2) Extracellular proteins produced without *C. flexuosus* essential oil treatment; Lane (3) Extracellular proteins with *C. flexuosus* essential oil treatmen; Lane (4) Extracellular proteins produced in combination of *C. flexuosus* essential oil treatment and pH media adjustment to pH 6; Lane (5) Extracellular proteins produced in combination of *C. flexuosus* essential oil treatment and pH media

adjustment to pH 7; Lane (6) Extracellular proteins produced in combination of *C. flexuosus* essential oil treatment and pH media adjustment to pH 8.

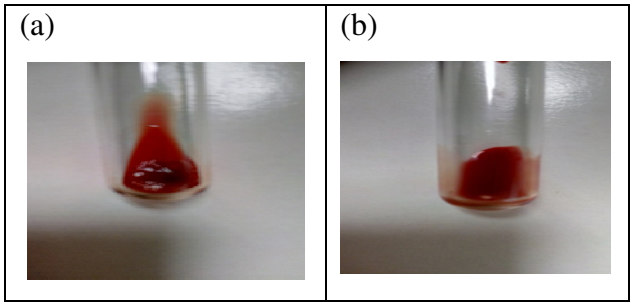

Fig. 2. Anti-coagulant Activity of Extracellular Protein Produced *B. subtilis* ATCC21332 in media withpH 6: (a) without*EO as stress inducer -blood coagulation occur (no anti-coagulant activity); (b) with EO as stress inducer -blood coagulation did not occur (showed anti-coagulant activity).Note: *EO = *C. flexuosus* Essential Oil.

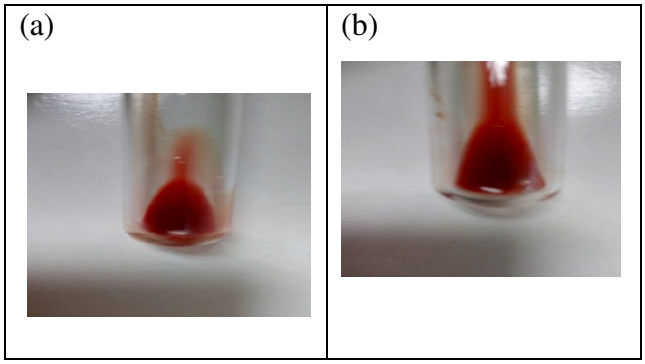

Fig. 3. Anti-coagulant Activity of Extracellular Protein Produced *B. subtilis* ATCC21332 in media with pH 7: (a) without *EO as stress inducer - blood coagulation did not occur (showed anti-coagulant activity); (b) with EO as stress inducer - blood coagulation did not occur (showed anti-coagulant activity). Note: *EO = *C. flexuosus* Essential Oil.

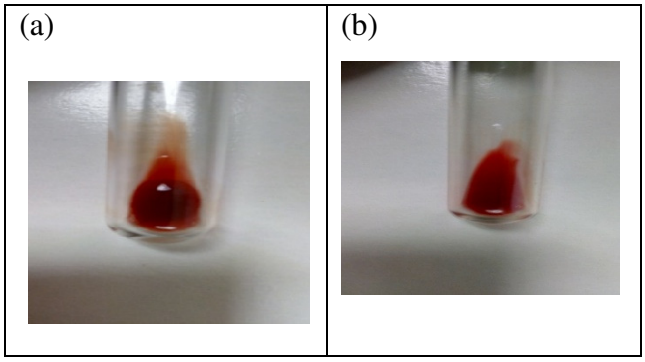

Fig. 4. Anti-coagulant Activity of Extracellular Protein Produced *B. subtilis* ATCC21332 in media with pH 8: (a) without *EO as stress inducer - blood coagulation occur (no anti-coagulant activity); (b) with EO as stress inducer - blood coagulation did not occur (showed anti-coagulant activity). Note: *EO = *C. flexuosus* Essential Oil.

DISCUSSION

Bacillus subtilis and related bacilli have the capacity to secrete a variety of proteins into their environment, at frequently high concentrations. This has led to the commercial exploitation of bacilli as cell factories for secreted enzymes. In soil, the natural habitats of *B. subtilis*, secreted proteases are frequently synthesized as part of an adaptive response to changes in the environment, allowing the cell to benefit optimally from the available resources. To respond to these changes, *B. subtilis* makes use of complex signal transduction systems to sense a wide variety of extracellular stimuli [19]. *B. subtilis* uses two-component signal transduction systems to sense intra- and extracellular stimuli to adapt to fluctuating environmental situations [20]. These two-component systems are involved in various processes, such as competence development, protein secretion, synthesis of peptide antibiotics and bacteriocins and sporulation [19].

Bacteria are often exposed to wildly fluctuating environmental stresses, including changes in temperature, pH, osmolarity, radiation and the concentration of nutrients and toxins. To ensure survival in the face of these adversities, bacteria may adapt to changes in their immediate vicinity by responding to the imposed stress. The response to 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 gene expression [21].

Study on effects of *C. flexuosus* essential oil towards protein synthesis by *B. subtilis* ATCC21332 cells was previously described. When the bacterial cells were cultured for 24 h and 48 h in the presence of 0.01 MIC of *C. flexuosus* essential oil, there was no any new protein produced. The bacterial cells tend to produced new proteins when incubation time was increased to 72 h. It showed that *B. subtilis* ATCC21332 cells could maintain their normal physiological function within 48 h treatment with *C. flexuosus* essential oil. The bacterial cells were induced to introduce new protein in order to overcome the environmental stress caused by *C. flexuosus* essential oil after 72 h of incubation. *C. flexuosus* essential oil in which was added to early exponentially of growing cells resulting the production of a new protein with approximate size of 30 kDa, recognized as Bacillopeptidase F after 72 h of incubation [16]. This protein was reported to show antithrombotic and fibrinolytic effects and could be used for human benefit in future [7].

In this present study, SDS-PAGE analysis exhibited a proteins profile with a band in approximate size of 30 kDa was appeared for the treated bacteria with *C. flexuosus* essential oil and cultivated at three difference pH of media. Based on the intensity of protein bands observed in SDS-PAGE analysis, *B. subtilis* ATCC21332 could be induced to express more protein or specifically Baccilopeptidase F after being induced with *C. flexuosus* essential oil and cultivated at three difference pH media.

One of the most noticeable responses of *B. subtilis* cells towards a range of stress and starvation conditions is the dramatic induction of several 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 could protect cells against heat, acid, alkaline or osmotic stress [22].

In recent years, in order to enhance the efficacy and safety of fibrinolytic therapy, much effort has been done to discover fibrinolytic enzyme from microorganisms, including *B. subtilis*.Bacillopeptidase F is one of the main microbial fibrinolytic enzymes isolated from *Bacillus* sp. in which might play an important role in preventing the thrombotic disease. It has the ability to degrade fibrin in blood clot or act as bloods anticoagulant [3]. Previous study reported that *B. subtilis* ATCC21332 in stressful condition with the presence of *C. flexuosus* essential oil at low concentration (0.01 MIC) could induce the production of protein with approximate size of 30 kDa recognized as Bacillopeptidase F [16].

In this study, the extracellular proteins secreted by *B. subtilis* ATCC21332 after being treated with 0.01 MIC of *C. flexuosus* essential oil and cultivated either at pH 6 or pH 7 or pH 8 could prevent blood clotting. However, the extracellular proteins produced by *B. subtilis* ATCC21332 without inducing with 0.01 MIC of *C. flexuosus* essential oil only exhibited anti-coagulant activity when the bacterial cells were cultured at pH 7. It showed that *B. subtilis* ATCC21332 could be induced to highly express Bacillopeptidase F protein after being induced with *C. flexuosus* essential oil and cultivated at three difference pH media. As a conclusion, *B. subtilis* ATCC21332 can be enhanced to produce anti-coagulant enzymes after being treated with low concentration of *C. flexuosus* essential oil and cultivated in natural pH media or even in slightly basic or slightly acidic environment. Further study

should be done to purify, identify and analyze the anti-coagulant enzymes from *B. subtilis* ATCC21332.

CONCLUSION

B. subtilis ATCC 21332 can be enhanced to produce anti-coagulant enzymes after being treated with low concentration of *C. flexuosus* essential oil and cultivated in neutral pH media or even in slightly basic or slightly acidic environment.

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