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Protein Production by *Bacillus Subtilis* Atcc 21332 in the Presence of *Cymbopogon* Essential Oils

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***Abstract*—**Proteins levels produced by bacteria may be increased in stressful surroundings, such as in the presence of antibiotics. 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. There have been comparatively few studies on the potential of antibiotics or natural compounds in nature as a specific chemical signal that can trigger a variety of biological functions. Therefore, this study was focusing on the effect of essential oils from *Cymbopogon flexuosus* and *C. nardus* in regulating proteins production by *Bacillus subtilis* ATCC 21332. The Minimum Inhibition Concentrations (MICs) of both essential oils on *B. subtilis* were determined by using microdilution assay, resulting 0.2% and 1.56% for each *C. flexuosus* and *C. nardus* subsequently. The bacteria were further exposed to each essential oils at concentration of 0.01XMIC for 2 days. The proteins were then isolated and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein profile showed that a band with approximate size of 250 kD was appeared for the treated bacteria with essential oils. Thus, *Bacillus subtilis* ATCC 21332 in stressful condition with the presence of essential oils at low concentration could induce the protein production.

Keywords*—Bacillus subtilis* ATCC 21332, *Cymbopogon* essential oils, protein

I. Introduction

MANY different organisms produce cationic peptides, often referred to as antimicrobial peptides [1]. *Bacillus sp*. produce a broad spectrum of bioactive peptides with great potential for biotechnological and biopharmaceutical application, including lipopeptides which act as biosurfactants and peptides antibiotics with potent antimicrobial activities [2-3].

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 [4]. Study showed that the proteins levels are increased for the bacteria to survive in stressful surroundings, such as in the presence of antibiotics [5].

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 signaling molecules [1]. Antimicrobial agents or antibiotics with different structures and modes of action at sub-minimal inhibitory cocentrations (sub-MICs) have the ability to cause global changes in gene transcription [6]. 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 [7]. 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. Streptophenazines showed an antimicrobial activity against *Bacillus subtilis* and *Staphylococcus lentus* [8].

It is well established that bacteria are exposed to and respond to many different extracellular signals in the environment. However, there have been comparatively few studies on the potential of antibiotics or natural compounds in nature as a specific chemical signal that can trigger a variety of biological functions. Therefore, this present study will focus on the roles of antimicrobial compounds (*Cymbopogon* essential oils) in regulating proteins or peptides production by bacteria, which is *Bacillus subtilis* ATCC 21332.

II. Materials And Methods

A. Essential Oils, Bacterial Strains and Culture Conditions

Essential oils extracted from *Cymbopogon nardus* and *C. flexuosus* were provided by Al-muqarram Holdings Sdn Bhd. *Bacillus subtilis* strain ATCC 21332, obtained from American Type Culture Collection (ATCC) were grown in Mueller-Hinton Broth (Oxoid, USA).

B. Minimum Inhibition Concentration (MIC) Determination

The minimum inhibitory concentration (MIC) of essential oils against *B. subtilis* ATCC 21332 was determined by the microdilution assay [9]. Serial dilutions of essential oils in MHB were prepared and added to cultures with 10^7 cells/ml of *B. Subtilis* ATCC 21332 at exponential phase of growth. The samples were then incubated for an overnight at 37°C. Viable bacterial were detected by addition of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., USA). The minimum concentration of essential

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oils inhibiting the growth of *B. Subtilis* was considered the MIC.

C. Protein Production

Bacillus subtilis ATCC 21332 cells were tested for their reactions to the presence of essential oils. Bacterial cells were maintained on Mueller-Hinton Broth (MHB) and transfered into 100 ml flaks containing 10 ml of MHB. The culture was shaken vigorously at 37°C for an overnight and 1 ml was taken to inoculate 50 ml of fresh MHB medium in 250 ml flasks. Essential oils (*C. nardus* or *C. flexuosus*) at a concentration of 0.01 MIC were added to the bacterial culture after 3 h of cultivation and the cultures were shaken vigorously at 37°C for 24 – 48 h. A culture to which essential oil was not added served as a control.

D. Protein extraction

Protein extraction was done according to method by [10]. The bacterial cells were separated from the suspension by centrifugation at 2,000 x *g* for 5 min at room temperature, followed by washing with phosphate-buffered saline (Cambrex Bioscience, Verviers, Belgium) twicely, before being dissolved in sterile distilled water. Portions were kept apart for protein assay. The suspensions were mixed 1:1 with Laemmli buffer (Bio-Rad, Singapore) heated at 95°C for 10 min, and cooled on ice.

E. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Protein Identification

Proteins were analyzed by electrophoresis on Tris-HCl ready gels with 10% cross polymer in a Protean III electrophoresis system (Bio-Rad, Hercules, CA) with benchmark prestain protein ladder (10-170 kDa) (Fermentas). The protein bands made visible by staining with Biosafe coomasive blue (Bio-Rad, USA) and the bands of interest were identified by amino acid sequencing. The sequences were screened for similarity to proteins in the NCBI BLAST database

III. Results

B. subtilis ATCC 21332 was grown in the presence of essential oils from *C. flexuosus* and *C. nardus* with various concentrations. The effect of various concentrations of both essential oils on cell growth was studied and the minimal inhibition concentration (MIC) was determined for performing the subsequent experiment. The bacterial viability was determined by using the MTT as an indicator. This colorant substance was added to the test solutions and incubated at 37ºC for 30 min. If there is any blue formazan was formed as shown in Fig. 1, showing that the bacterial growth occurred. The MIC value was taken as the highest dilution yielding no bacterial growth [9]. Essential oil from *C. flexuosus* could inhibit the growth of *B. subtilis* ATCC 21332 at low concentration, which was 0.2%. The other essential oil, isolated from *C. nardus* could also inhibit *B. subtilis* ATCC 21332 with MIC value of 1.56%.

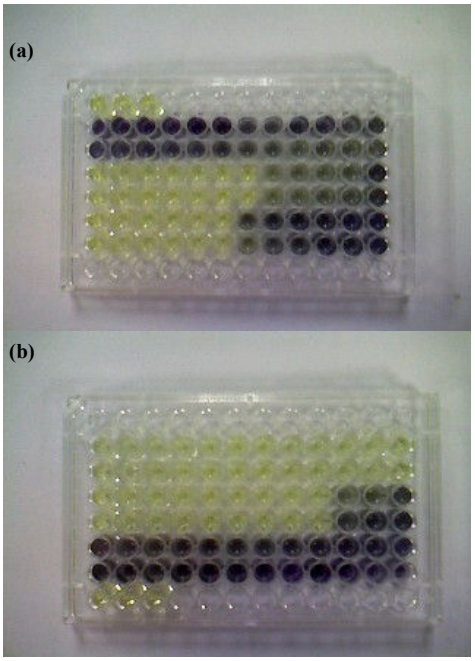

Fig. 1 Determination of MIC value by microdilution assay using MTT as an indicator: Treatment of (a) *C. nardus* essential oil and (b) *C. flexuosus* on *B. subtilis* ATCC 21332. The blue color showed bacterial growth due to the blue formazan formed, while the pale yellow indicated no bacterial growth

B. subtilis ATCC 21332 was grown in the presence of essential oils from *C. flexuosus* and *C. nardus*. The effects of these essential oils at concentration of 0.01 MIC on protein production were studied. When *B. subtilis* ATCC 21332 cells were grown in MHB for 24 h in the presence of 0.01 MIC essential oils, several protein bands with higher or lower expression levels than the control sample were detected on an SDS-PAGE gel (Fig. 2). Nevertheless, protein profile showed that there was no additional band appeared or new protein produced by *B. subtilis* ATCC 21332 after treatments either with *C. nardus* or *C. flexuosus* essential oils for 24 h. Therefore, the fermentation process or the time of incubation was increased to 48 h.

When *B. subtilis* ATCC 21332 cells were grown in MHB for 48 h in the presence of 0.01 MIC essential oils, several protein bands with higher or lower expression levels than the control sample were detected on an SDS-PAGE gel (Fig. 3). Besides, there was an additional band appeared or new protein produced by *B. subtilis* ATCC 21332 after treatments either with *C. nardus* or *C. flexuosus* essential oils for 48 h. Both essential oils induced the production of protein with similar size of 180 kDa.

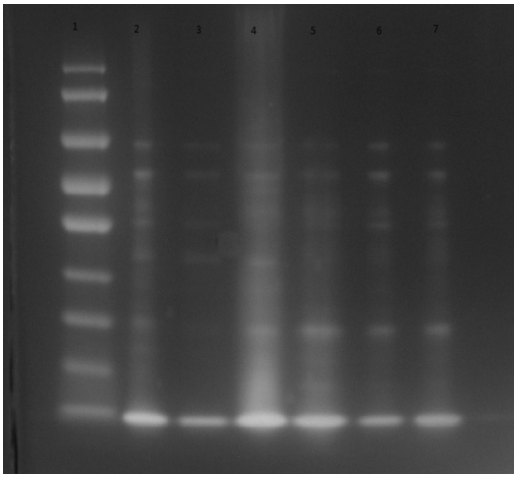

Fig. 2 SDS-PAGE of protein production by *Bacillus subtilis* ATCC 21332 after 24 h incubation: Lane (1) protein ladder (10-170 kDa); Lane (2) – (3) in the absence of essential oils (as a control); Lane (4) – (5) in the presence of *C. flexuosus* essential oil; Lane (6) – (7) in the presence of *C. nardus* essential oil

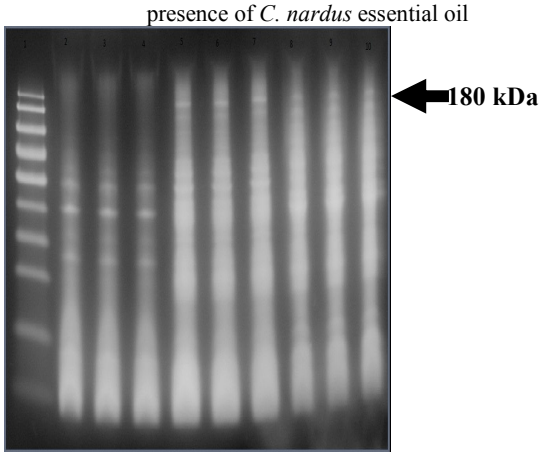

Fig. 3 SDS-PAGE of protein production by *Bacillus subtilis* ATCC 21332 after 48 h incubation: Lane (1) protein ladder (10-170 kDa); Lane (2) – (4) in the absence of essential oils (as a control); Lane (5) – (7) in the presence of *C. flexuosus* essential oil; Lane (8) – (10) in the presence of *C. nardus* essential oil. The arrow indicates respective protein band with the size of 180 kDa

Further analysis on the proteins produced after treatment with both essential oils was done by amino acid sequencing. The peptide sequences of proteins produced by *B. subtilis* ATCC 21332 after treatments with *C. flexuosus* and *C. nardus* are shown in Fig. 4 and Fig. 5 respectively. An alligment of peptide sequences to NCBI BLAST database revealed that different kind of protein was produced by *B. subtilis* ATCC 21332 after treatments with essential oils isolated from two different varieties of *Cymbopogon* sp. *B. subtilis* ATCC 21332 cells were tend to produce DNA-directed RNA polymerase β subunit enzyme *via* treatment with *C. flexuosus* essential oil. Whilst, respiratory nitrate reductase α subunit enzyme was produced by *B. subtilis* ATCC 21332 after treatment with *C. nardus* essential oil.

1 MTGQLVQYGR HRQRRSYARI SEVLELPNLI EIQTSSYQWF LDEGLREMFQ
51 DISPIEDFTG NLSLEFIDYS LGEPKYPVEE SKERDVTYSA PLRVKVRLIN
101 KETGEVKDQD VFMGDFPIMT DTGTFIINGA ERVIVSQLVR SPSVYFSGKV
151 DKNGKKGFTA TVIPNRGAWL EYETDAKDVV YVRIDRTRKL PVTVLLRALG
201 FGSDQEILDL VGENEYLRNT LDKDNTENSD KALLEIYERL RPGEPPTVEN
251 AKSLLDSRFF DPKRYDLANV GRYKINKKLH IKNRLFNQRL AETLVDPETG
301 EILAEKGQIL DRRTLDKVLP YLENGIGFRK LYPNGGVVED EVTLQSIKIF
351 APTDQEGEQV INVIGNAYIE EEIKNITPAD IISSISYFFN LLHGVGDTDD
401 IDHLGNRRLR SVGELLQNQF RIGLSRMERV VRERMSIQDT NTITPQQLIN
451 IRPVIASIKE FFGSSQLSQF MDQTNPLAEL THKRRLSALG PGGLTRERAG
501 MEVRDVHYSH YGRMCPIETP EGPNIGLINS LSSYAKVNR**F GFIETPYR**RV
551 DPETGKVTGR **IDYLTADEED NYVVAQANAR** LDDEGAFIDD SIVARFRGEN
601 TVVSRNRVDY MDVSPKQVVS AATACIPFLE NDDSNRALMG ANMQRQAVPL
651 MQPEAPFVGT GMEYVSGKDS GAAVICKHPG IVERVEAKNV WVRRYEEVDG
701 QKVKGNLDKY SLLKFVRSNQ GTCYNQRPIV SVGDEVVKGE ILADGPSMEL
751 GELALGRNVM VGFMTWDGYN YEDAIIMSER LVKDDVYTSI HIEEYESEAR
801 DTKLGPEEIT RDIPNVGEDA LRNLDDRGII RIGAEVKDGD LLVGKVTPKG
851 VTELTAEERL LHAIFGEKAR EVRDTSLRVP HGGGGIIHDV KVFNREDGDE
901 LPPGVNQLVR VYIVQKRKIS EGDKMAGRHG NKGVISKILP EEDMPYLPDG
951 TPIDIMLNPL GVPSRMNIGQ VLELHMGMAA RYLGIHIASP VFDGAREEDV
1001 WETLEEAGMS RDAKTVLYDG RTGEPFDNRV SVGIMYMIKL AHMVDDKLHA
1051 RSTGPYSLVT QQPLGGKAQF GGQRFGEMEV WALEAYGAAY TLQEILTVKS
1101 DDVVGRVKTY EAIVKGDNVP EPGVPESFKV LIKELQSLGM DVKILSGDEE
1151 EIEMRDLEDE EDAKQADGLA LSGDEEPEET ASADVERDVV TKE

Fig. 4 Peptide sequences of protein produced by *B. subtilis* ATCC 21332 *via* treatment with *C. flexuosus* essential oil.

1 MKKKKMSPLF RRLNYFSPIE HHSNKHSQTT REDRDWENVY RNRWQYDKVV
51 RSTHGVNCTG SCSWNIYVKN GIVTWEGQNL NYPSTGPDMP DFEPRGCPRG
101 ASFSWYIYSP LRVKYPYVRG VLINLWREAL QAHQNPLDAW KSIVENPEKA
151 KSYKQARGKG GFVRAEWPEV LKLISASLLY TVMKYGPDRN VGFSPIPAMS
201 MISHASGSRF MSLIGGPMLS FYDWYADLPP ASPQIWGDQT DVPESSDWYN
251 SGYIITWGSN VPLTR**TPDAH FLAEAR**YKGA KVISISPDFA ESSKFADDWL
301 SIRQGTDGAL AMAMGHVILQ EFYVNQETER FIEYAKQYTD FPFFVTLSKE
351 NGVYTAGRFL HAKDIGRQTK HDQWKPAVWN EQTSAFAIPQ GTMGSRWDGQ
401 QKWNLHMIDE ETGDPIEPRL SLLGIEDEIG TVRIPYFSND GNKVLERDLP
451 IKKLNLNGEE VCVTTVFDLI LANYGVNRGF GEQSAVSYDD PEPFTPAWQE
501 QMTGIKKEAV IKIAREFAQN AIDTDGRSMI IVGAGINHWF HSDTIYRAVL
551 NLVLLVGAQG VNGGGWAHYV GQEKLRPAEG WQTIANAKDW EGVPKLQNGT
601 SFFYFATDQW RYEDQPISDL ASPIAASSRY KHHADYNVLA ARLGWLPSYP
651 TFNQNGIDLY KEAEKAGATT PEDIGAYVAS QLQEKKLK**FA IEDPDNEVNF**
701 **PRNLFVWR**AN LISSSGKGHE YFLKHLLGTT NGLMNDDSDS IRPEEIKWRE
751 QAPEGKLDLL INLDFRMAGT ALYSDIVLPA ATWYEKHDLS STDMHPFIHP
801 FAPAISAPWE SKSDWDIFKA LSKAVSDLAE EVDMEPVKEV VATPLLHDTM
851 QELAQPFGKI NDWNKGECEA IPGKTMPNIQ VVERDYKRIF HKMTALGPHA
901 GLKPSGTKGM SWSIADEYES LKKRLGEITS DSVAKGCPNI SEAKQAAEAI
951 LTLSSTSNGK VAVKAWESLE NITNLKLKDL AEEREEECFT FEQITAQPKT
1001 VITSPAFTGS EKGGRRYSPF TTNVEKLIPW RTLTGRQSYY VDHELMMEFG
1051 ETMATFKPIL QHRPFLSKRP DQEGKEIVLN YLTPHNKWSV HSMYFDSLPM
1101 LTLFRGGPTV WMNKDDAEDT DIKDNDWIEC FNRNGVVVAR AVLSHRIPKG
1151 MAFMHHAQDR HINVPGTKLT NNRGGTHNSP TRIHVKPTQM IGGYAQLSYG
1201 FNYYGPTGNQ RDLNVVIRKL KEVDWLED

Fig. 5 Peptide sequences of protein produced by *B. subtilis* ATCC 21332 *via* treatment with *C. nardus* essential oil.

IV. Discussions

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 needs 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 [11; 12].

In this report, some effects of essential oils isolated from *C. flexuosus* and *C. nardus* on protein synthesis in *B. Subtilis* ATCC 21332 cells are described. When bacterial cells were cultured 24 h in the presence of 0.01 MIC essential oils, there was no any new protein produced. The bacterial cells tend to produced new proteins when incubation time was increased to 48 h. It showed that *B. subtilis* ATCC 21332 cells could maintain their normal physiological function within 24 h treatment with essential oils. The bacterial cells were induced to introduce new protein in order to overcome the

environmental stress caused by essential oils after 48 h incubation.

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 [11]. In this study, each of *C. flexuosus* and *C. nardus* essential oils was added to early exponentially of growing cells resulting the production of new proteins after 48 h incubation. Each treatment with *C. flexuosus* and *C. nardus* essential oils caused *B. subtilis* ATCC 21332 to synthesis the DNA-directed RNA polymerase β subunit enzyme and respiratory nitrate reductase α subunit enzyme respectively.

B. subtilis ATCC 21332 encounter drastic changes in their environment with the presence of *C. nardus* essential oil, resulting the production of respiratory nitrate reductase α subunit enzyme. *C. nardus* essential oil may induce the fluctuation of external oxygen level. The external oxygen limitation is sensed by bacterial cells and the bacterial cells then adjust their cellular metabolism to promote growth in an anaerobic environment. These changes are achieved by modulating protein activity, by regulating the expression of the appropriate genes, or both.

A two-component signal transduction system composed of a sensor kinase, ResE, and a response regulator, ResD, occupies an early stage in the regulatory pathway governing anaerobic respiration. One of the essential roles of ResD and ResE in anaerobic gene regulation is induction of *fnr* transcription upon oxygen limitation. *Fnr* is a transcriptional activator for anaerobically induced genes, including those for respiratory nitrate reductase, narGHJI. *B. subtilis* has two distinct nitrate reductases, one for the assimilation of nitrate nitrogen and the other for nitrate respiration [11]. Thus, essential oil may effect the activity of respiratory nitrate reductase produced by *B. subtilis* ATCC 21332 by regulating the expression of the narGHJI genes.

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]. Present study showed that in the presence of *C. flexuosus* essential oil as well as limited nutrients, *B. subtilis* ATCC 21332 cells tend to produce DNA-directed RNA polymerase β subunit enzyme.

The principal mechanism for control of gene expression is through regulation of the amount of mRNA produced from the corresponding gene. This is primarily determined by the affinity of RNA polymerase for the promoter. Genes that encode proteins which control basal transcription, including the five-subunit RNA polymerase core enzyme (α_2, β, β', ω) and σ-factors for binding specifically to different classes of promoters and hence selective expression of different groups of genes, are present in the bacterial genome [12]. The basis features of the transcriptional machinery are remarkably conserved in all organisms. In particularly, the β, β' and α subunits comprise the catalytic core of eubacterial RNA polymerase. Each RNA polymerase subunits was coded by different genes. For e.g *rpo*A gene codes for α subunit, while *rpo*B gene codes for β subunit [13, 14].

Rifampicin and streptolydigin are antibiotics which inhibit prokaryotic RNA polymerase at the initiation and elongation steps, respectively. In *Escherichia coli*, resistance to each antibiotic results from alterations in the β subunit of the core enzyme. However, in *B. subtilis*, reconstitution studies found rifampicin resistance (Rif^R) associated with the β subunit and streptolydigin resistance (Stl^R) with β' [14]. In the presence of essential oil which act as antimicrobial agent, *B. subtilis* ATCC 21332 may induce resistance by controlling the expression *rpo*B gene that encode the DNA-directed RNA polymerase β subunit enzyme.

V. Conclusion

Bacillus subtilis ATCC 21332 in stressful condition with the presence of either *C. flexuosus* or *C. nardus* essential oils at low concentration (0.01 MIC) could induced the protein production.

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