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Cymbopogon nardus Essential Oil as Protein Inducer in *Bacillus subtilis* ATCC21332

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Abstract*— Protein production by bacteria might be increased in stressful conditions such as in the presence of antimicrobial agents. Many studies have proven that antibiotics or antimicrobial agents at low concentration are able to activate or repress gene transcription process in bacteria. However, there have been comparatively few studies on the potential of natural compounds in nature as a specific chemical signal that can trigger a variety of biological functions. An attempt was made to study the effect of essential oil from *Cymbopogon nardus* in regulating protein production by *Bacillus subtilis* ATCC21332. The bacterial cells were further exposed to the *C. nardus* essential oil at concentration of 0.02 % for 48 h at 37°C. The intracellular proteins were then isolated and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins profile showed that a band with approximate size of 180 kDa appeared for the treated bacteria with *C. nardus* essential oil. An alignment of peptide sequences to the NCBI BLAST database revealed that *B. subtilis* ATCC21332 in stressful condition tend to produce intracellular protein recognized as respiratory nitrate reductase α subunit enzyme. Besides, the extracellular proteins secreted by *B. subtilis* ATCC21332 after being subjected to 0.02% of *C. nardus* essential oil for 48 and 72 h at 30°C, were further analyzed on antimicrobial activity. The extracellular proteins secreted by *B. subtilis* ATCC21332 prior to enhancing with 0.02 % *C. nardus* essential oil at 30°C for 72 h exhibited antimicrobial activity towards two strains of bacteria, which are *Bacillus cereus* and *Escherichia coli*.

***Keywords*— *Bacillus subtilis* ATCC21332; *Cymbopogon nardus* essential oil; protein.**

I. INTRODUCTION

Naturally, most of bacteria including *Bacillus subtilis* can produce proteins either intracellular or extracellular proteins at certain environmental conditions. Intracellular protein that act inside cells are responsible for catalysing the millions of reactions that occur in metabolic pathway such as glycolysis in the mitochondria and photosynthesis in the chloroplast [1]. Whilst, extracellular protein is the protein secreted by a cell and that works outside of the cell such as the human digestive enzyme, known as proteases [2].

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 [3]-[4]. However, under hostile and challenging environmental conditions, such as depletion of nutrients, oxygen, and changes in temperature, bacteria launch stress responses which improve their chances to adapt and survive to the various insults. Bacteria could develop a special physiologic mechanism for their survival by producing the specific proteins as well increasing the protein level production [5].

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 minimize the toxicity of the drug, as well as the activation of chaperones and the signal transduction cascade dedicated for sensing and responding to various stress [6]. Study showed that the protein 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 a weapon in microbial communities at high concentrations due to their inhibitory activity towards other microorganisms. In ecological environments, these compounds may be at lower concentrations and likely play additional roles as signalling molecules [7]. 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 [8].

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 [9]. 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 *B. subtilis* and *Staphylococcus lentus* [10].

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 natural compounds in nature as a specific chemical signal that can trigger a variety of biological functions. Therefore, this present study focused on the roles of natural antimicrobial compounds or specifically *C. nardus* essential oil in regulating intracellular proteins production by *B. subtilis* ATCC21332.

Escalating incidents of life-threatening infections by antibiotic-resistant bacteria in recent years have provided strong impetus to discover new antibiotics. Thus, this study was also aimed for future discovering of microbial proteins or peptides that have a potential as a novel source of antimicrobial substances. In this present study, an attempt was made to evaluate the antimicrobial activity of extracellular proteins secreted by *B. subtilis* ATCC21332 during mild stress condition in the presence of *C. nardus* essential oil.

II. Material and Method

A. Essential Oil, Bacterial Strains and Culture Conditions

Cymbopogon nardus essential oils were provided by Al-Muqarram Holdings Sdn Bhd. *Bacillus subtilis* strain ATCC21332, obtained from the American Type Culture Collection (ATCC) were grown in Mueller-Hinton Broth (Oxoid, USA). Test microorganisms used for antimicrobial screening include *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Escherichia coli*, *Klebsiella pneumonia and Shigella dysentriae*, obtained from ATCC were grown in Mueller-Hinton Broth (MHB; Oxoid, USA).

B. Intracellular Proteins Production and Extraction

Intracellular protein production and extraction were carried out based on the method proposed by [11]. The bacterial cells were harvested in 100 ml conical flask containing 10 ml of MHB with gentle agitation at 37°C. After an overnight incubation, 1 ml of bacterial culture was then transferred to 50 ml of fresh MHB medium in 250 ml conical flask before being shaken vigorously at 37°C. After 3 h of cultivation, 0.02 % *C. nardus* essential oil was then added to the bacterial culture. The culture was further incubated at 37°C for 48 h with gentle agitation. A culture to which essential oil was not added serve as a control.

After 48 h of fermentation at 37°C, 1 ml of each bacterial culture was transferred to 1.5 ml microcentrifuge tube and centrifuged at 2,000 x *g* for 5 min at room temperature (Microcentrifuge; Eppendorf, USA). The pellet cells which are intracellular proteins were then separated from fermentation medium and washed with phosphate-buffered saline (Cambrex Bioscience, Belgium) twice, before being dissolved in sterile distilled water. The protein suspension was 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 further analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

C. Extracellular Proteins Production and Extraction

Fermentation process for production of extracellular proteins was conducted based on the method proposed by [12]. In this process, a single colony of *B. subtilis* ATCC21332 was inoculated with 50 ml of MHB and incubated at 30°C with gentle agitation. *C. nardus* essential oil at concentration of 0.02 % was then added to bacterial culture after 8 h of cultivation and the culture was further shaken vigorously at 30°C for 48 h and 72 h. A culture to which essential oil was not added serve as a control.

The extracellular proteins from supernatant were extracted according to the method proposed by [13]. After each 48 h and 72 h of fermentation, the bacterial cells were separated from the supernatant by centrifugation at 900 x *g* for 15 min at 4°C (Microcentrifuge; Eppendorf, USA). The separated supernatant was then collected and transferred to a new tube. Approximately 80 % (w/v) of ammonium sulphate (Sigma-Aldrich, USA) was added to the supernatant for the protein precipitation process before being kept for 1 h at 4°C. The precipitated extracellular proteins were then collected by centrifugation at 15,000 x *g* for 20 min. The resulting pellet was resuspended in phosphate buffer solution (PBS, pH 6.8) before being further evaluated for antimicrobial activity.

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

The intracellular 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 (10-260 kDa) (Fermentas, USA). The protein bands made visible by staining with Biosafe^{TM} coomasive blue R-250 staining solution (Norgen Biotech Corporation, Canada) and the bands of interest were identified by amino acid sequencing. The sequences were screened for similarity to proteins in the NCBI BLAST database.

E. Antimicrobial Activity Screening via Well Diffusion Test

Antimicrobial activity of extracellular proteins against selected bacterial strains was determined by using agar well diffusion test based on [14]. Each bacterial culture at a concentration of 10^7 cell/ml was spread onto Muller-Hinton Agar (MHA; Oxoid, USA) plates. Wells with 6 mm diameter were then made with agar by using sterile borer. A 10 µl of each extracellular protein with different durations of fermentation was loaded into each respective well. The agar plates were then incubated at 37°C and the diameter of clear inhibition zones were measured after 24 h of incubation.

III. Results and Discussion

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 [15]-[16].

The essential oil from *C. nardus* is known as citronella oil, has been traditionally used as mosquito repellent, household

fumigant or fragrance agent in food commodities, soaps and cosmetics [17]. Besides, a study by [18] showed that *C. nardus* essential oil has antimicrobial activity against *S. aureus*, *S. epidermidis*, *B. subtilis*, *E. coli*, *S. typhimurium* and *S. marcescens*.

Several studies have shown that antimicrobial agents at sub-MIC or lower concentration were able to cause global changes in the bacterial gene transcription process [19], [20], [8], [21]. carter, davies, Henderson]. Besides, it has been reported that the protein levels are increased for the bacteria to survive in stressful surroundings, such as in the presence of antibiotics [5]. Therefore, attempt was made to study the effects of *C. nardus* essential oil at 0.02 % in enhancing protein production by *B. subtilis* ATCC21332.

There was a long-held belief that the Gram-positive soil bacteria, *B. subtilis* are strict aerobe. However, 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]. In this report, the effects of *C. nardus* at low concentration on protein production by *B. subtilis* ATCC21332 were studied. After 48 h of fermentation process at 37°C in the presence of 0.02 % *C. nardus* essential oil, the microbial proteins were isolated and analysed by SDS-PAGE. The SDS-PAGE analysis was conducted in order to further determine and identify any new protein produced by *B. subtilis* ATCC21332 after being induced with *C. nardus* essential oil. Based on the SDS-PAGE profile as shown in Figure 1, *B. subtilis* ATCC21332 introduced a new intracellular protein with approximate size of 180 kDa after being treated with *C. nardus* essential oil. Further analysis on the intracellular proteins produced by *B. subtilis* ATCC21332 prior to enhancing with *C. nardus* essential oil was done by amino acid sequencing. The peptide sequences of intracellular proteins produced by *B. subtilis* ATCC21332 after inducing with *C. nardus* essential oil are shown in Figure 2. An alignment of peptide sequence to NCBI BLAST database revealed that *B. subtilis* ATCC21332 in stressful condition (on the presence of *C. nardus* essential oil) tend to produce intracellular protein recognized as respiratory nitrate reductase α subunit enzyme.

B. subtilis ATCC21332 encounter drastic changes in their environment with the presence of *C. nardus* essential oil, resulting in 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 [15]. Thus, essential oil may affect the activity of respiratory nitrate reductase produced by *B. subtilis* ATCC21332 by regulating the expression of narGHJI genes.

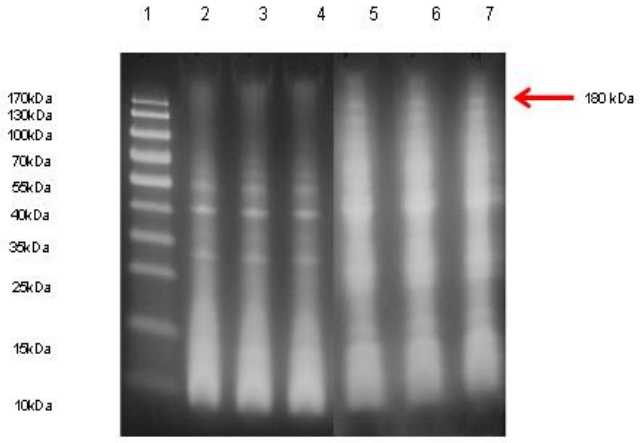

Fig. 1 SDS-PAGE profile for intracellular proteins produced by *B. subtilis* ATCC21332 in the presence of *C. nardus* after 48 h of incubation: Lane (1) protein ladder (10-170 kDa); Lane (2) – (4) in the absence of *C. nardus* essential oil (as a control); Lane (5) – (7) in the presence of C. nardus essential oil.

1 MKKKKMSPLFRRLNYFSPIE HHSNKHSQTT REDRDWENVY RNRWQYDKVV
51 RSTHGVNCTG SCSWNIYVKN GIVTWEGQNL NYPSTGPDMP DFEPRGCPRG
101 ASFSWYIYSP LRVKYPYVRG VLDNLWREAL QAHQNPLDAW KSIVENPEKA
151 KSYKQARGKG GFVRAEWPEV LKLISASLLY TVMKYGPDRN VGFSPIPAMS
201 MISHASGSRF MSLIGGPMLS FYDWYADLPP ASPQIWGDQT DVPESSDWYN
251 SGYIIT WGSN VPLTRTPDAH FLAEARYKGA KVISISPDFA ESSKFADDWL
301 SIRQGTDGAL AMAMGHVILQ EFVVNQETER FIEYAKQVTD FPFFVTLSKE
351 NGVYTAGRFL HAKDIGRQTK HDQWKPAVWN EQTSAFAIPQ GTMGSRWDGQ
401 QKWNLHMIDE ETGDPIEPRL SLLGIEDEIG TVRIPYFSND GNKVLERDLP
451 IKKLNLNGEE VCVTTVFDLI LANYGVNRGF GEQSAVSYDD PEPFTPAWQE
501 QMTGIKKEAV IKIARE FAQN AIDTDGRSMI IVGAGINHWF HSDTIYRAVL
551 NLVLLVGAQG VNGGGWAHYV GQEKLRPAEG WQTIANAKDW EGVPKLQNGT
601 SFFYFATDQW RYEDQPISDL ASPIAASSRY KHHADYNVLA ARLGWLPSYP
651 TFNQNGIDLY KEAEKAGATT PEDIGAYVAS QLQEKKLKFA IEDPDNEVNF
701 PRNLFVWRAN LISSSGKGHE YFLKHLLGTT NGLMNDDSDS IRPEEIKWRE
751 QAPEGKLDLL DNLDFRMAGT 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 EKGGGRYSPF TTNVEKLDW RTLTGRQSYY VDHELMMEFG
1051 ETMATFKPIL QHRPFLSKRP DQEGKEIVLN YLTPHNKWSV HSMYFDSLPM
1101 LTLFRGGPTV WMNKDDAEDT DIKDNDWIEC FNRNGVVVAR AVLSNRIPKG
1151 MAFMHHAQDR HINVPGTKLT NNRGGTHNSP TRIHVKPTQM IGGYAQLSYG
1201 FNYYGPTGNQ RDLNVVIRKL KEVDWLED

Fig. 2 Peptide sequences of intracellular protein produced by *B. subtilis* ATCC21332 *via* treatment with *C. nardus* essential oil.

The extracellular proteins isolated after the treatment with 0.02 % *C. nardus* essential oil were labelled as PN1 and PN2, each for 48 h and 72 h of fermentation time respectively. Whilst, the control protein samples (those that produced without prior treatment with 0.02 % *C. nardus* essential oil) were labelled as CN1 and CN2 for 48 h and 72 h subsequently. In this study, the antimicrobial activities of extracellular proteins were evaluated against different types of Gram-positive and Gram-negative bacterial strains, including *S. aureus*, *S. epidermidis*, *B. cereus*, *E. coli*, *K. pneumonia and S. dysentriae*. The spectrum of antimicrobial activity was determined according to size of inhibition zone formed as shown in Figure 3.

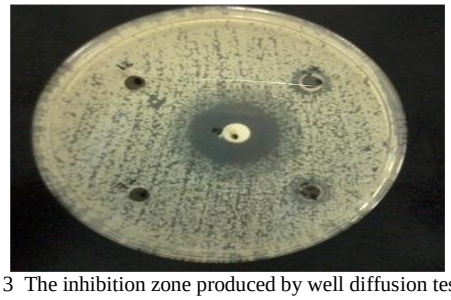

Fig. 3 The inhibition zone produced by well diffusion test

The results of antimicrobial activity for both extracellular proteins (PN1 and PN2) and control (CN1 and CN2) samples produced by *B. subtilis* ATCC21332 at 30 °C for 48 h and 72 h fermentation time are shown in Table 1. The extracellular protein produced by *B. subtilis* ATCC21332 after inducing with 0.02 % *C. nardus* essential oil at 30°C for 48 h (PN1) could not inhibit any of the bacterial strains tested. Nevertheless, PN2 which is the extracellular proteins produced by *B. subtilis* ATCC21332 after inducing with 0.02% *C. nardus* essential oil at 30 °C for 72 h exhibited antimicrobial activity towards *B. cereus* and *E. coli*. Meanwhile, the control samples (CN1and CN2) which are the proteins produced by *B. subtilis* ATCC21332 without inducing with 0.02% *C. nardus* essential oil did not show any antimicrobial activity against all the bacterial strains tested. Generally, Gram-positive bacteria are easy to be inhibited compared to Gram-negative bacteria. The Gram-negative organisms are less susceptible to the action of antimicrobials since they possess an outer layer of membrane which protects the cell wall. The cell wall restricts the hydrophobic compounds to diffuse through the lipopolysaccharide covering [22].

The extracellular proteins produced by *B. subtilis* ATCC21332 after inducing with 0.02% *C. nardus* essential oil for 48 h of fermentation at 30ºC showed no antimicrobial activity against any bacterial strains tested. It might be due to lack of any new extracellular proteins produced when the bacteria cells were induced with *C. nardus* essential oil. It showed that *B. subtilis* ATCC21332 has the ability to adapt and maintain its normal physiological function, hence could overcome the environmental stress caused by *C. nardus* essential oil within 48 h of incubation.

Nevertheless, the protein synthesized by *B. subtilis* ATCC21332 prior to enhancing with 0.02% of *C. nardus* essential after 72 h of fermentation at 30ºC could inhibit only two strains of bacteria which are *B. cereus* and *E. coli*. This low antimicrobial activity could be related to the presence or production of new intracellular protein which is respiratory nitrate reductase α subunit enzyme after the bacterial cells have been induced with *C. nardus* essential oil. However, other factors should be considered such as the pH of the medium, incubation period, temperature and cell density. These factors would be affected the enhancement of antimicrobial protein production [23].

TABLE I
SPECTRUM OF ANTIMICROBIAL ACTIVITY OF EXTRACELLULAR PROTEINS PRODUCED BY *B. subtilis* ATCC21332 PRIOR TO ENHANCING WITH *C. nardus* ESSENTIAL OIL

Test/Control Samples	**Test Microorganisms**					
	B. cereus	***K. pneumonia***	***S. dysenteriae***	***S. aureus***	***S. epidermidis***	***E. coli***
[a]PN1 (48h)	-	-	-	-	-	-
PN2 (72h)	[f]+	-	-	-	-	+
[b]CN1 (48h)	[g]-	-	-	-	-	-
CN2 (72h)	-	-	-	-	-	-
[c]MHB + EO	-	-	-	-	-	-
[d]MHB	-	-	-	-	-	-
[e]S	+	+	+	+	+	+

[a]PN1-PN2 Protein produced by *B. subtilis* after inducing with 0.01 MIC of *C. nardus* essential oil, each at 48h and 72h of incubation time; [b]CN1-CN2 Protein produced by *B. subtilis* without inducing with 0.01 MIC of *C. nardus* essential oil, each at 48h and 72h of incubation time; [c]MHB+ EO MHB with *C. nardus* essential oil as negative control; [d]MHB as negative control; [e]Streptomycin sulfate as a positive control; [f] +inhibition zone; [g]- no inhibition zone.

IV. Conclusions

The present study is done to explore the effects of *C. nardus* essential oil at low concentration (0.02 %) in inducing the production of proteins by *B. subtilis* ATCC21332. This study discovered that *B. subtilis* ATCC21332 in harsh environmental conditions with the presence of 0.02 % *C. nardus* essential oil was able to produce the intracellular proteins which are recognized as respiratory nitrate reductase α subunit enzyme. However, *B. subtilis* ATCC21332 in this mild stress environment could only secrete the extracellular proteins with a low antimicrobial activity. Therefore, other related factors such as pH of the medium, incubation period, temperature and cell density in which could affect the enhancement of antimicrobial protein production should be considered for further research.

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References

[1] Y. Kim, M. Coppey, R. Grossman, L. Ajuria, G. Jimmenez, S. Paroush and Shvartsman., "Antibacterial activity of some essential oil components against five foodborne pathogens," *Current Biology*, vol.20, pp.1-6, 2010.

[2] G. K. Sims, "Nitrogen starvation promotes biodegradation of N-heterocyclic compounds in soil," *Soil Biology and Biochemistry*, vol. 38, pp. 2478-2480, 2006.
[3] M. M. Al-Ajlani, S. M. Abid, Z. Ahmad and S. Hasnain, "Production of surfactin from *Bacillus subtilis* MZ-7 grown on pharmamedia commercial medium," *Microbial Cell Factories*, vol. 6, pp. 1-8, 2007.
[4] V. M. Leclere, A. Bechet, J-S. Adam, B. Guez, M. Wathelet,P. Ongena, F. Thonart, M. Gancel, and P. Chollet-Imbert Jacques, "Mycosubtilin overproduction by *Bacillus subtilis* BBG100 enhances the organism's antagonistic and biocontrol activities," *Applied and Environmental Microbiology*, vol. 71, pp. 4577-4584, 2005.
[5] M., T. Tanaka, A. Hasegawa, K. Torii Okamoto, and M. Ohta, "Effect of Antibiotics on Group A *Streptococcus* Exoprotein Production Analyzed by Two-Dimensional Gel Electrophoresis," *Antimicrobial Agents and Chemotherapy*, vol. 49, pp. 88-96, 2005.
[6] L. E. Cowen and W. J. Steinbach., "Stress, Drugs, and Evolution: the Role of Cellular Signaling in Fungal Drug Resistance," *American Society for Microbiology*, vol. 7, pp. 747-764, 2008.
[7] G. Yim, H. H. Wang and F.R.S. J. Davies, "Antibiotics as signalling molecules," *Philosophical Transactions of the Royal Society Biological Sciences*, vol. 362, pp. 1195-1200, 2007.
[8] J. Davies, G. B. Spiegelman and G. Yim., "The world of subinhibitory antibiotic concentrations," *Current Opinion in Microbiology*, vol. 9, pp. 445-453, 2006.
[9] E. B. Goh, G. Yim, W. Tsui, J. McClure, M. G. Surette and J. Davies., "Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics," in *Proceedings of the National Academy of Sciences of the United States of America*, 2002, vol. 99. pp. 17025-17030.
[10] M. I. Mitova, G. Lang, J. Wiese and J. F. Imhoff., "Subinhibitory concentrations of antibiotics induce phanazine production in a marine *Streptomyces* sp.," *Journal of Natural Product*, vol. 71, pp. 824-827, 2008.
[11] S.A. R. van deer Zee Burt, A. P. Koets, A. M. de Graaff, F. van Knapen, W. Gaastra, H. P. Haagsman and J. A. Veldhuizen., "Carvacrol induces heat shock protein 60 and inhibits synthesis of flagellin in *Escherichia coli* 0157:H7," *Applied and Environmental Microbiology*, vol. 73, pp. 4484-4490, 2007.
[12] B. W. Lash, T. H. Mysliwiec and H. Gourama., "Detection and partial characterization of a broad-range bacteriocin produced by *Lactobacillus plantarum* (ATCC 8014)," *Food Microbiology*, vol. 22, pp. 199-204, 2005.
[13] S. C. Chang, and L. J. Cseke., "Extraction and purification of proteins," *Handbook of Molecular and Cellular in Biology and Medicine*, vol. 3, pp. 48-49, 2004.
[14] D. Kalemba and A. Kunicka., "Antibacterial and antifungal properties of essential oils," *Current Medicinal Chemistry*, vol. 10, pp. 813-829, 2003.
[15] M. M. Nakano and P. Zuber., "Anaerobic Growth of a "Strict Aerobe" (*Bacillus Subtilis*)," *Annual Review of Microbiology*, vol. 52, pp. 165-190, 1998.
[16] S.KP. Lau, R. Y.Y. Fan, T. C.C. Ho, G. KM. Wong, A. KL. Tsang, J. L.L. Teng, W. Chen, R. M. Watt, S. O.T. Curreem, H. Tse, K. Y. Yuen and P. C.Y. Woo, "Environmental adaptability and stress tolerance of *Laribacter hongkongensis*: a genome-wide analysis," *Cell & Bioscience*, vol. 1, pp. 22, 2011.
[17] K. Nakahara, N. J. Alzoreky, T. Yoshihashi, H. T. T. Nguyen and G. Trakoontivakorn, "Chemical Composition and Antifungal Activity of Essential Oil from *Cymbopogon nardus* (Citronella Grass)," *Japan Agricultural Research Quarterly*, vol. 37, pp. 249-252, 2003.
[18] M. Zakiah, "Antimicrobial activity of essential oils from *Cymbopogon nardus*," BSc thesis, Universiti Sains Islam Malaysia, Nilai, Malaysia, 2008.
[19] R. P. Adhikari and R. P. Novick, "Subinhibitory cerulenin inhibits Staphylococcal exoprotein production by blocking transcription rather than by blocking secretion," *Microbiology*, vol. 151, pp. 3059-3069, 2005.
[20] G. Carter, L. S. Young and L. E. Bermudez, "A subinhibitory concentration of clarithromycin inhibits *Mycobacterium avium* biofilm formation," *Antimicrobial Agents and Chemotheraphy*, vol. 48, pp. 4907-4910, 2004.
[21] S. K. Henderson-Begg, D. M. Livermore and L. M. Hall, "Effect of subinhibitory concentrations of antibiotics on mutation frequency in *Streptococcus pneumoniae*," *Journal of Antimicrobials and Chemotheraphy*, vol. 57, pp. 849-854, 2006.
[22] M. Vaara, "Agents that increase the permeability of the outer membrane," *Microbiology Review*, vol. 56, pp. 395, 1992.
[23] K. Vijayalakshmi and S. Rajakumar, "Antimicrobial protein production by *Bacillus amyloliquefaciens* MBL27: An application of statistical optimization technique," *African Journal of Microbiology Research*, vol. 4, pp. 2388-2396, 2010.