International Journal of Biological, Life and Agricultural Sciences
ISSN: 2415-6612
Vol:8, No:8, 2014

Extracellular Protein Secreted by *Bacillus subtilis* ATCC21332 in the Presence of Streptomycin Sulfate

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***Abstract*—**The extracellular proteins secreted by bacteria may be increased in stressful surroundings, such as in the presence of antibiotics. It appears that many 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 as a specific chemical signal that can trigger a variety of biological functions. Therefore, this study was carried out to determine the effect of Streptomycin Sulfate in regulating extracellular proteins secreted by *Bacillus subtilis* ATCC21332. Results of Microdilution assay showed that the Minimum Inhibition Concentration (MIC) of Streptomycin Sulfate on *B. subtilis* ATCC21332 was 2.5 mg/ml. The bacteria cells were then exposed to Streptomycin Sulfate at concentration of 0.01 MIC before being further incubated for 48h to 72 h. The extracellular proteins secreted were then isolated and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins profile revealed that three additional bands with approximate sizes of 30 kDa, 22 kDa and 23 kDa were appeared for the treated bacteria with Streptomycin Sulfate. Thus, *B. subtilis* ATCC21332 in stressful condition with the presence of Streptomycin Sulfate at low concentration could induce the extracellular proteins secretion.

Keywords*—Bacillus subtilis* ATCC21332, Streptomycin Sulfate, extracellular proteins.

I. Introduction

NATURALLY, most of bacteria including *B. subtilis* can secrete proteins, known as extracellular proteins at certain environment condition. Extracellular protein is the enzyme secreted by a cell that works outside of the cell. For instance, the enzyme in human digestive system like proteases that functions outside the cell from which it originates. Proteases hydrolyze the peptide bonds in proteins and therefore break down the proteins into their constituent monomers [1]. These proteins are particularly important in bacterial pathogenesis and have a range of biological functions ranging from host cell toxicity to more subtle alterations of host cell for the benefits of the invader [2].

Bacillus subtilis is an endospore forming bacteria and produces several antibiotics like Subtilosin, Bacitracin, Difficidin, Fengycin, Mersacidin, Bacilysocin and Iturin. These antimicrobial compounds are effective against both Gram-positive and Gram-negative bacteria. Besides, *B. subtilis* has an ability to grow in extreme environments such as Alkaliphilic (high pH condition) or Halophilic (high salt condition) [3].

Environmental stress can be defined as an external factor that has an adverse effect on the physiological welfare of bacterial cells, leading to reduction in growth rate, or in more extreme circumstances, to inhibition or death, at individual cell or population levels. Examples of such bacteriostatic or bactericidal stresses include extremes of temperature, pH, osmotic pressure, depletion of nutrients, and the presence of toxic or inhibitory compounds, including antibiotics [4]. In order to adapt and survive in these environments, bacteria need the capability of protecting DNA damages and survive these extreme and rapidly changing conditions. Thus, the bacteria must sense the changes and then respond with appropriate alterations in gene expression and protein activity [5].

The minimum inhibition concentration (MIC) of an antibiotic is defined as the lowest concentration of the compound that completely inhibits the initiation of growth of a particular bacterium under standardized *in vitro* conditions. It appears that many antibiotics, when used at low concentrations or sub-MIC level, have in common the ability to activate or repress gene transcription, which is distinct from their inhibitory effect including morphological changes, modifications of cell wall structure, altered growth kinetics, inhibition of enzyme or toxin production, and loss of adhesive properties [6], [7].

Antibiotics could modulate the transcription of bacteria in a dose dependent manner. Furthermore, each antibiotic triggers a specific response, and those responses may have adaptive values. It has been suggested that antibiotics may have a role as signalling molecules besides inhibitors [8]. Study showed that the proteins levels are increased for the bacteria to survive in stressful surroundings, such as in the presence of antibiotics [9].

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 Streptomycin Sulfate as a selected antibiotic drug in regulating extracellular proteins secretion by *B. subtilis* ATCC21332.

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II. Materials and Methods

A. Streptomycin Sulfate, Bacterial Strains and Culture Conditions

Streptomycin Sulfate was provided by Duchefa Biochemie Sdn Bhd. *Bacillus subtilis* strain ATCC21332, 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 Streptomycin Sulfate against *B. subtilis* ATCC21332 was determined by the Microdilution assay [10]. Serial dilutions of Streptomycin Sulfate were prepared and added to cultures with 10^7 cells/ml of *B. subtilis* ATCC21332 at exponential phase of growth. The samples were then incubated for an overnight at 37°C. The bacterial viability was detected by using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., USA) as an indicator. The lowest concentration of Streptomycin Sulfate inhibiting the growth of *B. subtilis* was considered as the MIC value.

C. Protein Production

Bacillus subtilis ATCC21332 cells were tested for their reactions to the presence of Streptomycin Sulfate. A bacterial colony was inoculated in 50 ml of MHB before being further shaken vigorously at 30°C. Streptomycin Sulfate at concentration of 0.01 MIC was then added to bacterial culture after 8 h of cultivation (which is log or exponential phase) and the culture was further shaken vigorously at 30°C for 48 h to 72 h. A culture to which Streptomycin Sulfate was not added served as a control.

D. Protein Extraction

Protein extraction was done according to method by [11]. The bacterial cells were separated from the suspension by centrifugation at 900 x g for 15 min at 4ºC. The supernatant 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 4ºC. 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 4ºC 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. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Protein Identification

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) and the bands of interest were identified by amino acid sequencing. The sequences were screened for similarity to proteins by using the NCBI BLAST database.

III. Results

B. subtilis ATCC21332 was grown in the presence of Streptomycin Sulfate at concentration ranging from 0.0025 mg/ml to 10.0 mg/ml. The effects of this antibiotic on bacterial cell growth were studied in order to determine the minimal inhibition concentration (MIC) 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. As shown in Fig. 1, the blue formazan was formed due to the bacterial growth. The MIC value was determined as the highest dilution or lowest concentration yielding no bacterial growth. Streptomycin Sulfate could inhibit the growth of *B. subtilis* ATCC21332 at low concentration, which is 2.5 mg/ml.

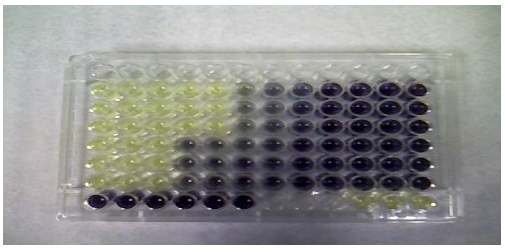

Fig. 1 Determination of MIC value by Microdilution assay using MTT as an indicator: Treatment of Streptomycin Sulfate on *B. subtilis* ATCC21332. The blue color showed bacterial growth due to the blue formazan formed, while the pale yellow indicated no bacterial growth

The effects of Streptomycin Sulfate at concentration of 0.01 MIC on extracellular proteins secretion were studied and analysed by SDS-PAGE. When *B. subtilis* ATCC21332 cells were grown in MHB for 48 h in the presence of 0.01 MIC Streptomycin Sulfate, several protein bands with higher or lower expression levels than the control sample were detected on SDS-PAGE gel (Fig. 2). Nevertheless, the protein profile showed that there was no additional band appeared or new extracellular proteins secreted by *B. subtilis* ATCC21332 after the treatment with Streptomycin Sulfate for 48 h.

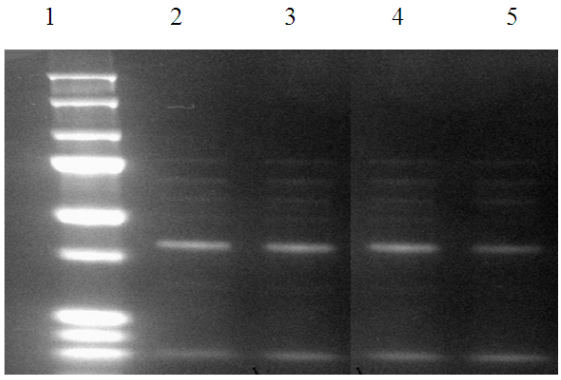

Fig. 2 SDS-PAGE analysis on protein production by *B. subtilis* ATCC21332 after 48 h incubation: Lane (1) protein ladder (2 kDa to 250 kDa); Lane (2) and (3) in the absence of Streptomycin Sulfate (as a control); Lane (3) and (4) in the presence of Streptomycin Sulfate

When the fermentation process or the time of incubation was increased to 72 h, several protein bands with higher or lower expression levels than the control sample were also detected on a SDS-PAGE gel (Fig. 3). Besides, there were additional bands appeared or new extracellular proteins secreted by *B. subtilis* ATCC21332 prior to treatment with Streptomycin Sulfate for 72 h. It showed that there are three new protein bands appeared on SDS-PAGE gel with approximate sizes of 30 kDa, 22 kDa and 23 kDa respectively. Despite of inducing the production of new proteins, deletion of one protein band with approximate size of 27 kDa could also be observed in Fig. 3.

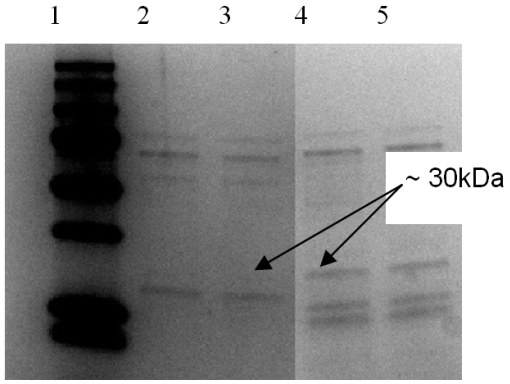

Fig. 3 SDS-PAGE analysis on protein production by *B. subtilis* ATCC21332 after 72 h incubation: Lane (1) protein ladder (2 kDa to 250 kDa); Lane (2) and (3) in the absence of Streptomycin Sulfate (as a control); Lane (4) and (5) in the presence of Streptomycin Sulfate

Further analysis on 30 kDa of extracellular proteins produced by *B. subtilis* ATCC21332 after inducing with Streptomycin Sulfate was done by Electrospray Mass Spectrometry (LC/MS/MS) and amino acid sequencing. An alligment of peptide sequences to NCBI BLAST database revealed that *B. subtilis* ATCC21332 in stressful condition (in the presence of Streptomycin Sulfate) tend to produce protein, recognized as Bacillopeptidase F (Fig. 4).

1 MKMKKKTKNR LISSVLSTVV ISSLLFPGAA GASSKVTSPS VKKELQSAES
51 IQNKISSSLK KSFKKKEKTT FLIKFKDQAN TEKAAKAAVK KAKSKKLSAA
101 KTEYQKRSAV VSSLKVTADE SQQDVLKYLN TQKDKGNADQ IHSYYVVNGI
151 AVHASKEVME KVAQFPEVEK VLPNEKRQLF KSSSPFNMKK AQKAIKATDG
201 VEWNVDQIDA PKAWALGYDG TGTVVASIDT GVEWNHPALK EKYRGYNPEN
251 PNEPENEMNW YDAVAGEASP YDDLAHGTHV TGTMVGSEPD GTNQIGVAPG
301 AKWIAVKAFS EDGGTDADIL EAGEWVLAPK DAEGNPHPEM APDVVNNSWG
351 GGSGLDEWYR DMVNAWRAAD IFPEFSAGNT DLFIPGGPGS IANPANYPES
401 FATGATDINK KLADFSLQGP SPYDEIKPEI SAPGVNIRSS VPGQAYEDGW
451 DGTSMAGPHV SAVAALLKQA NASLSVDEME DILTSTAEPL TDSTFPDSPN
501 NGYGHGLVNA FDAVSAVTDG LGKAEGQVSV EGDDQEPPVY QHEKVTEAYE
551 GGSLPLTLTA EDNVSVTSVK LSYKLDQGEW TEITAKRISG DHLKGTYQAE
601 IPDIKGTKLS YKWMIHDFGG HVVSSDVYDV TVKPSITAGY KQDFETAPGG
651 WVASGTNNNW EWGVPSTGPN TAASGEKVYG TNLTGNYANS ANMNLVMPPI
701 KAPDSGSLFL QFKSWHNLED DFDYGYVFVL PEGEKNWEQA GVYNGKTSSW
751 TDEEIDLSAY KGQNIQVMFN LQSDESIAKE GWYIDDVVLS DKSAGKTVKK
801 NKLGVEKPSG KQKKKPVNPK KAKPSANTAV KHQNKAIQPQ VLPLKAQVSV
851 VETGKSTYSD QSTGQYTLKH KAGDYTLMAE AYGYQSKTQK VSLKTDQTTQ
901 ANFTLEEMKK GTLKGTVINK TTGEPVTGAS VYVVEDAAVE PAMTNDKGEY
951 MLEAYEGAYT IKVAAPGYYS DEFSVELKGD VTKETALKPF VGYPGEIAYD
1001 DGTAENANSY FAAGNGWAVK MTLADGKDKG MLTGGLFRFW DTEFPDPGGT
1051 EFKVEVYDAT GKDGAPGKKI AGPFNAEALR NGEWTKVDLS SKGIMVDKDF
1101 YLVYIQSKPD PYSPGLAMDE TGQNSGRNWQ YIDGKWQPGD KADGNYMIRA
1151 LVDYEAAVPE ITSPTDKSYT NKDSVTVKGN ASPGTTVHIY NGEKEAGETK
1201 AAADGTFHAG IILNKGENEL TATASTDNGT TDASSPITVT LDQEKPELTL
1251 DNPKDGGKTN KETLTVKGAV SDDNLKDVKV NGKKATVADG SYSARILLEN
1301 GRNEIKVIAT DLAGNKTTKK TVIDVNFDKP VISGLIPGED KNLKAGESVK
1351 IAFSSAEDLD ATFTIRMPLT NARASVQNAT ELPLREISPG RYEGYWTATS
1401 SIKAKGAKVE VIVRDDYGNE TRKTANGKLN MNTEN

Fig. 4 Peptide sequences of protein produced by *B. subtilis* ATCC21332 *via* treatment with Streptomycin Sulfate

IV. Discussions

The ecological and evolutionary roles of antibiotics have been usually inferred from their therapeutically activity. Since those compounds inhibit bacterial growth, it was thought that they should be produced by soil microorganisms to inhibit the growth of competitors in natural habitats. However, it has been shown that antibiotics modulate transcription of bacteria in a dose dependent manner [8]. Sensing and adapting to fluctuations in environmental conditions are essential processes for the survival of all bacterial cells. The microbial communities are in flux and dependent on the changes in the environmental stresses, including exposure to antimicrobial agents. However, bacteria have developed many different mechanisms to survive in nutrient-depleted and harsh environments, varying from producing a more resistant vegetative cell to complex developmental programmes [7].

Streptomycin Sulfate is an antibiotic compound, precipitates nucleic acids, inhibits protein synthesis and interferes with proofreading, and thus causes translational errors. Streptomycin Sulfate isolated from *Streptomyces griseus* was found to be active against a wide variety of Gram-positive and Gram-negative bacteria [12]. In this study, the inhibitory effects of Streptomycin Sulfate at different concentration ranging from 0.0025 mg/ml to 10.0 mg/ml against *B. subtilis* ATCC21332 were evaluated by Microdilution assay. The effects of Streptomycin Sulfate towards bacterial growth were explored in order to determine the minimal inhibition concentration (MIC). The Microdilution assay is a technique used to determine the lowest concentration of an antimicrobial agent that could prevent or inhibit the growth of microorganism. The bacterial viability was evaluated by using 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) solution as an indicator. This analysis is a colorimetric assay that measures the reduction of yellow MTT by mitochondrial succinate

dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple or blue) formazan product. Since reduction of MTT can only occur in metabolically active cells, the colour changes can be used to evaluate the viability of the cells. The MIC value was determined as the highest dilution or lowest concentration of antimicrobial or inducer that showed no bacterial growth [10]. The MIC value of Streptomycin Sulfate against *B. subtilis* ATCC 21332 was recorded as 2.5 mg/ml.

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 [13]. In this report, effects of Streptomycin Sulfate on extracellular proteins secretion by *B. Subtilis* ATCC 21332 cells during fermentation process at 30°C are described. When bacterial cells were cultured for 48 h in the presence of 0.01 MIC Streptomycin Sulfate, there was no any new extracellular proteins produced. The bacterial cells tend to secrete new extracellular proteins when incubation time was increased to 72 h. It showed that *B. subtilis* ATCC 21332 cells could maintain their normal physiological function within 48 h treatment with Streptomycin Sulfate. The bacterial cells were induced to secrete the new extracellular proteins in order to overcome the environmental stress caused by Streptomycin Sulfate after 72 h of incubation. There are three new protein bands appeared on SDS-PAGE gel with approximate sizes of 30 kDa, 22 kDa and 23 kDa respectively. Despite of inducing new proteins, deletion of one protein band with approximate size of 27 kDa could also be observed. As reported by previous study using *C. flexuosus* essential oil as a bacterial stress inducer [14], Streptomycin Sulfate could also induce the secretion of new extracellular proteins with approximate size of 30 kDa.

Streptomycin Sulfate is the antibiotic which acts in the cells by interfering with the protein synthesis. It works by attaching onto a highly specific site on the surface of a bacteria's ribosome, where decoding of DNA information to proteins occurs. When the Streptomycin attaches, it stops this machinery from producing the right proteins, and the bacteria die [15]. When this antibiotic exist in very low concentration (for example at 0.01 MIC), it didn't kill the *B. subtilis* ATCC21332 cells, instead the bacteria cells need to adapt to this mild stress condition by modifying the protein synthesis to ensure the survival of the bacterial cells. Such event is one of the example how bacteria become resistant to antibiotic.

In this present study, Streptomycin Sulfate was added during log phase of growing cells, resulting in the production of new extracellular proteins after 72 h of incubation at 30°C. In this mild stress environment (in the presence of Streptomycin Sulfate at 0.01 MIC), *B. subtilis* ATCC 21332 cells were induced to secrete extracellular proteins with approximate size of 30 kDa, recognized as Bacillopeptidase F. Other study reported that the Bacillopeptidase F was produced and isolated from *B. subtilis natto* after 2 days of incubation at 42°C [16]. The temperature used was higher compared to the optimum growth temperature for *B. subtilis* which is about 30°C to 37°C [17]. It showed that *B. subtilis* could only produce or secrete Bacillopeptidase F in stressful condition or environment. Bacillopeptidase F is one of the minor proteases secreted by *B. subtilis* and other species [18]. The Bacillopeptidase F is synthesized by *B. subtilis* as a preproenzyme, and that small proteins are generated through processing at both the amino and carboxyl termini [19].

Although proteases are generally believed to help cells survive in adverse environments by providing peptides and amino acids from denatured or unnecessary proteins, the exact role and contribution of each enzyme is largely unknown [18]. Besides, more parameters should be tested to better understand the influence of growth temperature, pH of the medium and incubation time to the effectiveness of antimicrobial agents or antibiotic drug to act as an inducer for *B. subtilis* ATCC21332 to produce Bacillopeptidase F. Thus, the optimum condition could be further evaluated in order to produce the highest concentration of this serine protease.

Bacteria are not only submitted to potentially stressful environmental changes in industrial processes, but also in nature where the ability to quickly respond to stress is essential for survival. This current research explores the secretion of extracellular proteins by *Bacillus subtilis* ATCC 21332 in stressful condition. The study discovered that *B. subtilis* ATCC21332 in harsh environmental conditions with the presence of Streptomycin Sulfate at low concentration (0.01 MIC) were able to secrete the extracellular proteins, identified as Bacillopeptidase F. Therefore, further study can be done to isolate, purify and optimize the production of bioactive extracellular proteins (which is Bacillopeptidase F) as well as to determine and evaluate the biological activities of this protein.

V. Conclusion

B. subtilis ATCC21332 in the presence of Streptomycin Sulfate at low concentration were able to secrete the extracellular proteins, recognized as Bacillopeptidase F. Therefore, further study can be done to isolate, purify and optimize the production Bacillopeptidase F as well as to determine and evaluate the biological activities of this protein.

Acknowledgment

The authors acknowledge the Ministry of High Education Malaysia for the financial support through RACE Grant Scheme USIM/RACE/FST/STH/35/50412), RAGS Grant Scheme (USIM/RAGS/FST/STH/36/50212) and facilities through Universiti Sains Islam Malaysia.

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