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Preliminary Study of Surfactin Production by Malaysian Local Isolates of *Bacillus Subtilis*

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Abstract: Surfactin is one of the most powerful lipopeptidebiosurfactants produced by various strains of *Bacillus subtilis*. It has exceptional surface activity, with antiviral, antibacterial, and antitumor properties. The four local isolates, which were named *Bacillus subtilis*1M, 3M, 7M, and 8M were provided by the School of Biosciences and Biotechnology, Faculty of Science and Technology, University of Kebangsaan, Malaysia. In this study, fermentation on shaker flasks was carried out to assess the ability of four local isolates of *Bacillus subtilis* strains to produce surfactin by using Cooper's media formulation, and comparing their production with a commercial strain of *Bacillus subtilis* ATCC 21332, which was obtained from the American Type Culture Collection. High-Performance Liquid Chromatography (HPLC) was used for surfactin identification and surfactin concentration measurements. Results obtained show the four local isolates have the ability to produce surfactin. The *Bacillus subtilis*3M strain showed the highest amount of surfactin production with 117 (3) mg/L, while the *Bacillus subtilis*1M strain produced the lowest amount with 65 (5.4) mg/L. In addition, the production of *Bacillus subtilis* ATCC 21332 strain was found at 101(4) mg/L under the same fermentation conditions.

دراسة أولية لإنتاج مادة مضادة للتوتر السطحي *(Surfactin)* قوي للغاية نتج من العزلات المحلية الماليزية من البكتيريا العصوية الرقيقة

الكلمات المفتاحية :
بكتريا عصويه رقيقه؛
إنتاج surfactin؛
كروماتوجرافي سائل عالي الأداء (HPLC)؛
مقياس الطيف الضوئي الحيوي.

المستخلص: Surfactin هو واحد من أقوى المواد الخافضة للتوتر السطحي الدهني التي تنتجها سلالات مختلفة من *Bacillus subtilis*. له نشاط سطح استثنائي فعال، مع خصائص مضادة للفيروسات، والبكتيريا، ومضادة للأورام. تم توفير العزلات المحلية الأربعة التي تم تسميتها باسم *Bacillus subtilis* 1M و3 M و7 M و8 M من قبل كلية العلوم البيولوجية والتكنولوجيا الحيوية، كلية العلوم والتكنولوجيا، جامعة كيبانغسان ماليزيا. في هذه الدراسة تم إجراء التخمير على دورق شاكر لتقييم قدرة أربع عزلات محلية من سلالات العصيات الرقيقة على إنتاج سيرفاكتين باستخدام تركيبة وسط كوبر، ومقارنة إنتاجها بالسلالة التجارية من العصوية الرقيقة ATCC 21332، والتي تم الحصول عليها من سلالات العصوية الرقيقة. مجموعة الثقافة الأمريكية. تم استخدام الكروماتوغرافيا السائلة عالية الأداء (HPLC) لتحديد سيرفاكتين، وقياسات تركيز سيرفاكتين. أظهرت النتائج المتحصل عليها أن العزلات المحلية الأربع لديها القدرة على إنتاج Surfactin. أظهرت سلالة *Bacillus subtilis* 3M أعلى كمية إنتاج للسيرفاكتين مع 117 (3) مجم / لتر، بينما أنتجت سلالة *Bacillus subtilis* 1M أقل كمية مع 65 (5.4) مجم / لتر. بالإضافة إلى ذلك، تم العثور على إنتاج سلالة *Bacillus subtilis* ATCC 21332 عند 101 (4) ملجم / لتر تحت نفس ظروف التخمير.

INTRODUCTION

Bacillus subtilis, one of the most studied gram-positive bacteria (Li et al., 2010), is a sporulating rod bacteria that thrives in the soiland is non-pathogenic to human beings (Zweers et al., 2008). The ability of *Bacillus subtilis* to produce a lipopeptide has been documented for over 50 years (Waewthongrak et al., 2014), and the research

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on its antibacterial effectsis still ongoing (Fahle et al., 2022). The antibacterial properties of surfactinaredue to the ability of *Bacillus subtilis* to produce a variety of antibacterial agents(Cheng et al., 2018), which includes a broad spectrum of potent biosurfactants lipopeptides (Sumi et al., 2015).

The target spectrum of potent biosurfactants lipopeptidesproduct in this study is surfactin, known as one of the most effective surface activeagents available (Sousa et al., 2014). It has the ability to reduce the surface tension of water from 72 to 27 mN m^{-1} at a trace concentration as low as 0.005% (Amit & Rukhsar, 2013). This number is significantly lower than most biosurfactant surface tension data, as reported by (Al-Bahry et al., 2013). Surfactinis a cyclic lipopeptide consisting of a heptapeptideheadgroup with the sequence of L-Glu, L-Leu, D-Leu, L-Val, L-Asp, D-Leu, and L-Leu, closed to a lactone ring by a $C_{13–15}$ β-hydroxy fatty acid (Isa et al., 2007). In addition to high surface activity, surfactinhassseveral other attractive properties (Pereira et al., 2013), which include fibrin-clotting inhibition, antibiotic, antiviral, hemolytic, and antitumor properties (Chen et al., 2008). Due to these properties, surfactin was found to have the potential to be applied in biotechnology and medicine. This prospect makes surfactin production and application very attractive economically (Jiao et al., 2017) .Surfactin utilization for various applications in terms of critical micelle concentration (CMC) and minimum surface tension will increase the high cost of capital investment (Ríos et al., 2017). Furthermore, surfactinisnot widely utilized in industry due to high production costs associated with using expensive substrates and strains (dos Santos et al., 2010), suggested that several factors, such as microbes, the microbial growth substrate or feedstock processing, and by-product recovery, may influence biosurfactant production costs. During the last four decades,more research has been carried out to minimize the cost of surfactin production by improving its yield.(Zhi et al., 2017), managed to produce surfactin of 50-100 mg/L after 24 hours of culture. Jajor 2016 used a mineral salts' medium as fermentation media and achieved a surfactinyield of 780 mg/L with continuous product removal and metal cation addition.

(Mulligan et al., 1989), found an ultraviolet mutant of *Bacillus subtilis* ATCC 21332, which produced over three times more surfactin (1124 mg/L).In 1997, Senand Swaminathano ptimized the fermentation medium and obtained a maximum surfactin production of 760 mg/L. Another discovery (Wei & Chu, 2002) used an inorganic salt-enriched medium accompanied by appropriate pH control and managed to produce a surfactin yield of nearly 3500 mg/L, which seemed ready for commercialized applications. These latter efforts made it possible to reduce the costofsurfactin production. This study aimed to assess the ability of four local *Bacillus subtilis* strains, namely (1M, 3M, 7M, and 8M) to produce surfactin and compare their production with a commercial strain of *Bacillus subtilis* ATCC 21332 obtained from the American Type Culture Collection.

MATERIALS AND METHODS

Local isolates of *Bacillus subtilis*strains, namely 1M, 3M, 7M, and 8M, were obtained from the Biosciences and Biotechnology lab, Faculty of Science and Technology, University Kebangsaan Malaysia. The commercial strain of *Bacillus subtilis* ATCC 21332 was obtained from the American Type Culture Collection. At 4^{o}C, bacterialstrains were maintained on nutrient agar (NA). Tow loopfuls of colonies were inoculated in 100 ml of Cooper's medium, composed of 4% (w/v) glucose and mineral salt medium (Jajor et al., 2016) in 200 ml Erlenmeyer flasks and wereincubated in an incubator shaker at 200 rpm for 24 h at 30^{o}C. 0.5 ml of the seed culture was then inoculated into 200 ml of Cooper media in 500 ml Erlenmeyer flasks (Wei & Chu, 2002).

Determination of Bacterial Growth: The determination of bacterial concentration was referred to the method proposed by (Amit & Rukhsar, 2013). Cellular growth was expressed in terms of optical density at 600 nm (OD_{600nm}), measured by a Bio-spectrophotometer (Eppendorf, Germany).

Quantitative Analysis of Surfactin: To determine the concentration of surfactin, culture samples were withdrawn aseptically and centrifuged at 10 000 × g for 10 min and then filtered through a 0.45 µm Nylon membrane filter. The surfactin concentration was measured by using High-Performance Liquid Chromatography (HPLC) (Agilent Technologies, 1200 Series, USA) equipped with a C-18 column (Agilent Zorbax Eclipse C18, 250 mm × 4.6 mm, 5µm), and detected at 205 nm with a Variable Wavelength Detector (VWD). The system was run in isocratic mode at a flow rate of 1.5 mL/min with a mobile phase of 3.8 m Mtrifluoroacetic acid (TFA) in 80% acetonitrile. As a standard, surfactin from *Bacillus subtilis* with 98% purity from Sigma was used.

Statistical Analyses: The collected data were subjected to a mean analysis, a standard deviation of the mean value, and a level of significance determination using the Student's t-test. The differences between measurements were considered significant at the level of ($P< 0.05$).

RESULTS AND DISCUSSION

Several *Bacillus subtilis*strains are inherently defective in surfactin biosynthesis due to a frame-shift mutation in the *sfp* gene (Abushady et al., 2005) .*Bacillus subtilis*-strains were grown using a chemically defined mineral salt (MS) medium. In 1981 the medium was designed to promote surfactin production and was proposed by Cooper (Jajor et al., 2016). Environmental factors play a significant role in the yield and characteristics of the produced surfactin. In order to obtain large quantities of surfactin it is necessary to optimize the process conditions because the production of surfactin may be induced by changes in pH, incubation temperature, aeration, or agitation speed. Results obtained in this study showthe four local isolates of*Bacillus subtilis*namely (1M, 3M, 7M, and 8M), have the ability to produce surfactin at 30^{o}C, 200 rpm for 168 hours in a mineral medium containing 4% (w/v) glucose.

Time Course of Surfactin Production and Cell Growth: Figure1(A) and Figure1 (B) show the growth curve and surfactin production of *B. subtilis*1M, 3M, 7M, 8M, and a commercial strain of ATCC 21332 using Cooper medium, which was composed of 4% (w/v) glucose and mineral salt medium (MSM). Production of surfactin for *Bacillus subtilis*3M and 7M started at the log phase of fermentation and continues until 144 hours of fermentation. Maximum surfactin production in both strains was achieved in 96 hoursof fermentation in the range of 117 (±3)mg/L - 86 (±11) mg/L. Maximum cell growth was achieved in 72 hours of fermentation. Referring to Figure1 (B), maximum surfactin production was achieved early in the exponential phase.For *Bacillus subtilis*8M, surfactin production started at the log phase of fermentation and continued until 144 hours of fermentation, as shown in Figure 3. Maximum surfactin production was achieved at around 72 hours of fermentation, with a surfactin yield of 105 (±12) mg/L. Referring to Figure1 (A), the highest bacterial growth was at 72 hours of fermentation and was similar to results obtained by (Abdel-Mawgoud et al., 2008) for the production of surfactin by *Bacillus subtilis* Isolate BS5. Surfactinproduction started after 24 hours and wascontinues for144 hoursfor-*Bacillus subtilis*1M. The highest concentration of surfactin with 65 (±7) mg/L was achieved at 72 hours of fermentation, as shown in Figure 1(A),and cell growth remains almost stationary from 48 to 144 hours of fermentation. Referring to Figure 1(B), maximum surfactin production was achieved during the stationary phase. Surfactin production

for *Bacillus subtilis* ATCC 21332 startsat the log phase of fermentation and continues until 144 hours. As shown in Figure 1(A), the highest surfactin production was at 96 hours of fermentation with 101(±4) mg/L.Cell growth remains almost stationary from 48 to 144 hours of fermentation. Referring to Figure 1(B), the highest surfactin production was during the stationary phase. Surfactin production rises during the first 96 hours of incubation and falls after 120 hours. The amount produced was found to be quite similar to the previous work done by (Wei & Chu, 2002), with the highest amount of surfactin of 100 mg/L. Surfactin production was reported to begin at the late log phase and the early stationary phase of bacterial growth when nutrients in culture media become scarce (Kinsinger et al., 2005). Overall, the results obtained show *Bacillus subtilis*3M produced the highest amount of surfactin, whereas *Bacillus subtilis*1M produced the lowest amount of surfactin. In addition, the production of *Bacillus subtilis* ATCC 21332 was found quite similar to *Bacillus subtilis*3M under the same fermentation conditions. Statistical analysis shows the surfactin production of *Bacillus subtilis* ATCC 21332 strain was significantly higher ($P \leq 0.01$) in comparison to *Bacillus subtilis*1M, whereas there was no significant difference between *Bacillus subtilis* ATC*C* 21332 and *Bacillus subtilis*3M ($P \geq 0.05$).

Chromatographic Characterization: The High-Performance Liquid Chromatography (HPLC) assay used in this research work for surfactin identification and quantification was highly sensitive and reproducible. The identification and quantification of surfactin are complex since surfactin has a number of isoforms (Wei & Chu, 2002).

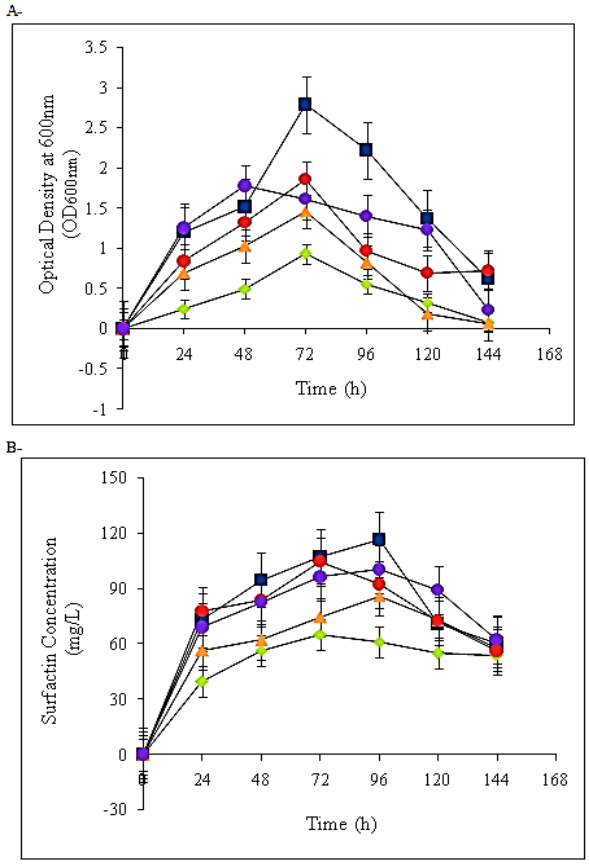

Figure (1). Time course of bacterial growth (A) and surfactin concentration (B):*Bacillussubtilis* 1M (♦);*Bacillussubtilis* 3M (■);*Bacillussubtilis*7M (▲);*Bacillussubtilis*8M (●);*BacillussubtilisATCC*21332 (●)

Previous studies by (Wei et al., 2003), demonstrated that surfactin has six isoforms, while others stated that so far, at least nine different surfactin isoforms have been identified (Abdel-Mawgoud et al., 2008). These isoforms differ in the length of the -hydroxy fatty acid chain, which is typically C13 to C15 (Vater et al., 2002).Therefore, HPLC analysis was performed in this study on crude fermentation broth to identify different surfactin isoforms that may be present. The surfactin standard chromatogram as shown in Figure 2, shows eleven different isoforms, which were eluted between 8 and 38 min. Of these isoforms, six were major namely, peaks 1, 2, 4, 5, 7, and 8, and five were minor namely, peaks 3, 6, 9, 10, and 11. In addition, the figure shows that all surfactin isoforms were well separated except for isoforms numbers 7

and 8, which differed in their retention times by less than 0.5 min, so they appeared somewhat merged. Surfactin chromatogram in Figure 3shows *Bacillus subtilis* 3M produced nine different surfactin isoforms and overall showsthe closest similarity to surfactin standard isoforms in Figure 2. The surfactin isoforms in Figure 3(designated by letters) show very similar retention times compared to the surfactin standard in Figure 2(designated by numbers), except for peaks8 and 9 in the surfactin standard, which were undetected inthesurfactin chromatogram of *Bacillus subtilis* 3M.

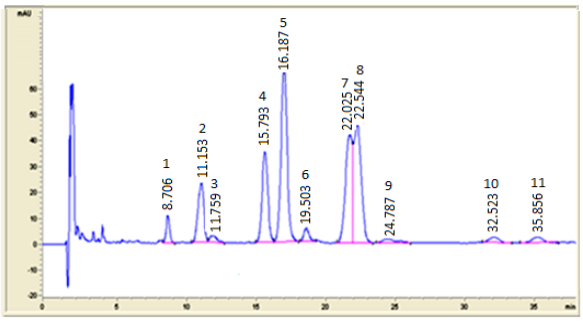

Figure (2). HPLC chromatogram of surfactin standard from Sigma

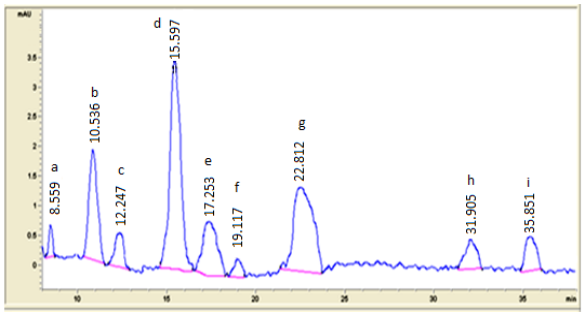

Figure (3). HPLC chromatogram of *Bacillus subtilis*3M

Bacillus subtilis 1M produced the least number of surfactin isoforms with only four, whereas *Bacillus subtilis* ATCC 21332as shown in Figure 4, *Bacillus subtilis* 8M, and 7M in Figure 6, Figure 5, and Figure 7, respectively produced in the range of six to seven different surfactin isoforms. It appeared that the number of surfactin isoforms produced and the relative abundance of the different isoforms were not the same in both the local isolates and *Bacillus subtilis* ATCC 21332, which may be related to strain variations. (Campos et al., 2013) stated that surfactin consists of a family of lipopeptides with similar chemical structures, i.e., isoforms, which are slightly different in their physicochemical properties due to variations in the chain length and branching of its hydroxy fatty acid component as well as substitutions of the amino acid components of the peptide ring. These differences depend on the strain variation and the nutritional and environmental conditions (Abdel-Mawgoud et al., 2008).

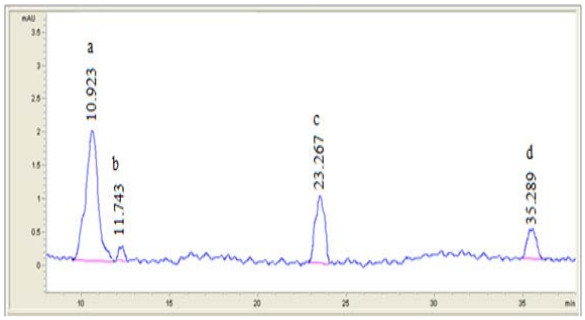

Figure (4). HPLC chromatogram of Bacillus subtilis 1M

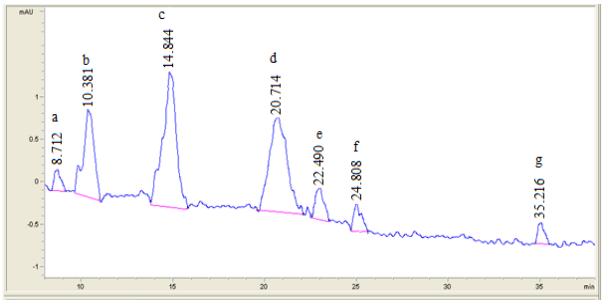

Figure (5). HPLC chromatogram of Bacillus subtilis 8M

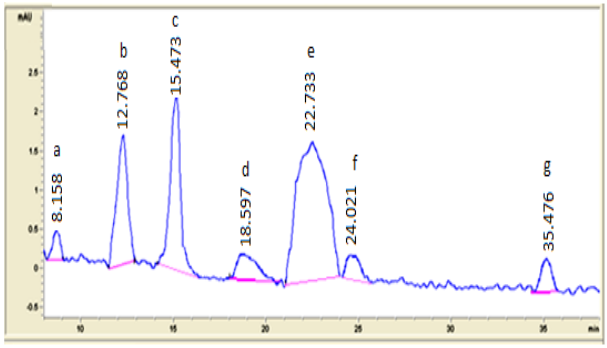

Figure (6). HPLC chromatogram of *Bacillus subtilis*ATCC21332

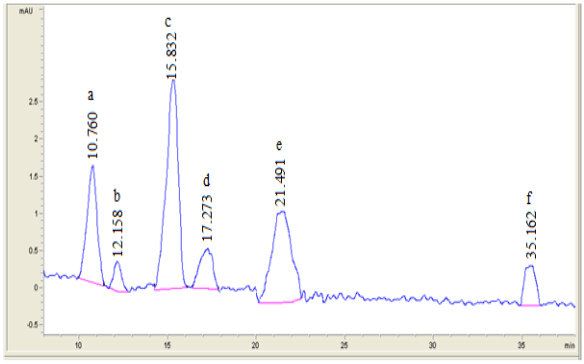

Figure (7). HPLC chromatogram of Bacillus *subtilis* 7M

CONCLUSION

The main objective of this research is to study the production of surfactin by using local isolates of *Bacillus subtilis*. This study shows the four local *Bacillus subtilis*isolates namely (1M, 3M, 7M, and 8M), have the ability to produce surfactin at 30ºC in a mineral medium containing 4% (w/v) glucose. Surfactin-HPLC chromatogram of four local isolates of *Bacillus subtilis*overall shows a similar profile of surfactin isoforms in comparison to the surfactin standard and surfactin produced by the ATCC 21332 strain, only differing slightly in terms of abundance of individual peak retention times and the number of isoforms. In addition, the *Bacillus subtilis*3M strain has the highest capacity to produce surfactin under facultative aerobic conditions, with a maximum production of 117 mg/L, and the-*Bacillus subtilis*1M strain produced the lowest amount of surfactin at 65 mg/L, at the same fermentation condition.

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