



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

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<b>ARTICLE HISTORY</b>	<b>Abstract:</b> Surfactin is one of the most powerful lipopeptidebiosurfactants produced by various strains of <i>Bacillus subtilis</i> . It has exceptional surface activity, with antiviral, antibacterial, and antitumor properties. The four local isolates, which were named <i>Bacillus subtilis</i> 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 <i>Bacillus subtilis</i> strains to produce surfactin by using Cooper's media formulation, and comparing their production with a commercial strain of <i>Bacillus subtilis</i> 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 <i>Bacillus subtilis</i> 3M strain showed the highest amount of surfactin production with 117 (3) mg/L, while the <i>Bacillus subtilis</i> 1M strain produced the lowest amount with 65 (5.4) mg/L. In addition, the production of <i>Bacillus subtilis</i> ATCC 21332 strain was found at 101(4) mg/L under the same fermentation conditions.
Received: 31 July 2022	
Accepted: 22 November 2022	
<b>Keywords:</b> <i>Bacillus subtilis</i> ; Surfactin Production ; High-Performance LiquidChromatography (HPLC); Bio- Spectrophotometer.	

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

<b>الكلمات المفتاحية :</b> بكتريا عنصوية رقيقة؛ إنتاج surfactin؛ كروماتوجرافي سائل عالي الأداء (HPLC)؛ مقياس الطيف الضوئي الحيوي.	<b>المستخلص:</b> Surfactin هو واحد من أقوى المواد الخافضة للتوتر السطحي الدهني التي تنتجها سلالات مختلفة من <i>Bacillus subtilis</i> . له نشاط سطح استثنائي فعال، مع خصائص مضادة للفيروسات، والبكتيريا، ومضادة للأورام. تم توفير العزلات المحلية الأربعة التي تم تسميتها باسم <i>Bacillus subtilis</i> 1M و 3M و 7M و 8M 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 العنصيات الرقيقة على إنتاج سيرفاكتين باستخدام تركيبة وسط كوبر، ومقارنة إنتاجها بالسلالة التجارية من العنصوية الرقيقة ATCC 21332، والتي تم الحصول عليها من سلالات العنصوية الرقيقة. مجموعة الثقافة الأمريكية. تم استخدام الكروماتوجرافيا السائلة عالية الأداء (HPLC) لتحديد سيرفاكتين، وقياسات تركيز سيرفاكتين. أظهرت النتائج المتحصل عليها أن العزلات المحلية الأربع لديها القدرة على إنتاج Surfactin. أظهرت سلالة <i>Bacillus subtilis</i> 3M أعلى كمية إنتاج للسيرفاكتين مع 117 (3) مجم / لتر، بينما أنتجت سلالة <i>Bacillus subtilis</i> 1M أقل كمية مع 65 (5.4) مجم / لتر. بالإضافة إلى ذلك، تم العثور على إنتاج سلالة <i>Bacillus subtilis</i> ATCC 21332 عند 101 (4) ملجم / لتر تحت نفس ظروف التخمير.
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## 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 effects is still ongoing (Fahle et al., 2022). The antibacterial properties of surfactin are due 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 lipopeptide product in this study is surfactin, known as one of the most effective surface active agents available (Sousa et al., 2014). It has the ability to reduce the surface tension of water from 72 to 27 mN m<sup>-1</sup> 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). Surfactin is a cyclic lipopeptide consisting of a heptapeptide head group 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<sub>13-15</sub>  $\beta$ -hydroxy fatty acid (Isa et al., 2007). In addition to high surface activity, surfactin has several 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, surfactin is not 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 surfactin yield 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 Swaminathan optimized 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 cost of surfactin 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°C, bacterial strains were maintained on nutrient agar (NA). Two loops 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 were incubated in an incubator shaker at 200 rpm for 24 h at 30°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 Biospectrophotometer (Eppendorf, Germany).

**Quantitative Analysis of Surfactin:** To determine the concentration of surfactin, culture samples were withdrawn aseptically and centrifuged at  $10\,000 \times g$  for 10 min and then filtered through a  $0.45 \mu m$  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,  $250 mm \times 4.6 mm, 5\mu m$ ), 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 Trifluoroacetic 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

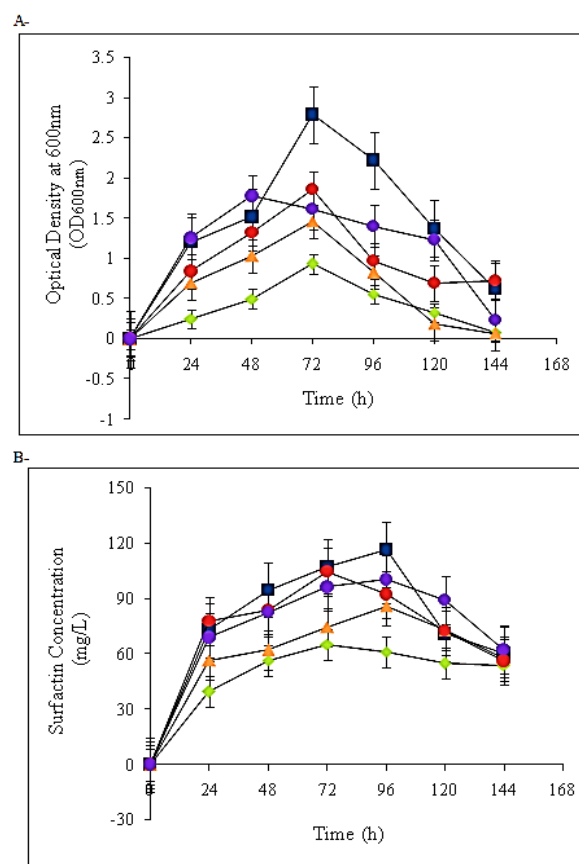
Several *Bacillus subtilis* strains are inherently defective in surfactin biosynthesis due to a frame-shift mutation in the *sfp* gene (Abushady et al., 2005). *Bacillus subtilis* strains were grown using a chemically defined mineral salt (MS) medium. In 1981 the medium was designed to promote surfactin 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 neces-

sary 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 show the four local isolates of *Bacillus subtilis* namely (1M, 3M, 7M, and 8M), have the ability to produce surfactin at  $30^{\circ}C$ , 200 rpm for 168 hours in a mineral medium containing 4% (w/v) glucose.

**Time Course of Surfactin Production and Cell Growth:** Figure 1(A) and Figure 1(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 hours of fermentation in the range of  $117 (\pm 3) mg/L$  -  $86 (\pm 11) mg/L$ . Maximum cell growth was achieved in 72 hours of fermentation. Referring to Figure 1(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  $105 (\pm 12) mg/L$ . Referring to Figure 1(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. Surfactin production started after 24 hours and was continued for 144 hours for *Bacillus subtilis* 1M. The highest concentration of surfactin with  $65 (\pm 7) mg/L$  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 starts at 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  $101(\pm 4)$  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* ATCC 21332 and *Bacillus subtilis* 3M ( $P \geq 0.05$ ).

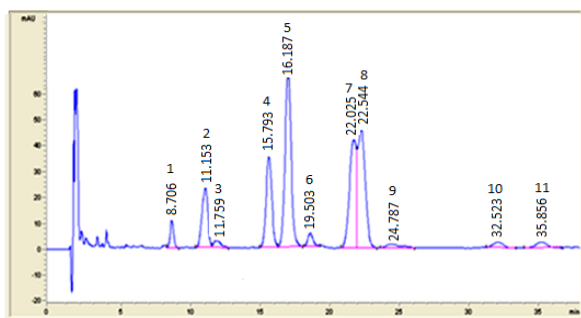
**Chromatographic Characterization:** The High-Performance Liquid Chromatography (HPLC) assay used in this research work for surfactin identification and quantification was highly sensitive and reproducible. The identification and quantification of surfactin are complex since surfactin has a number of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



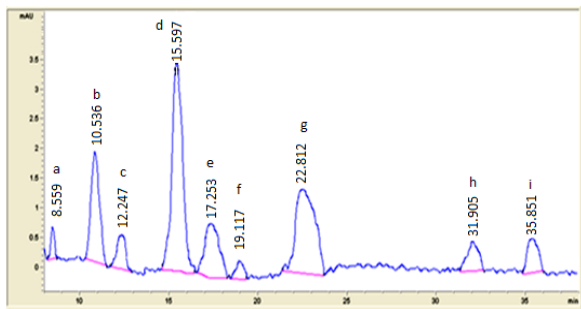
**Figure 1.** Time course of bacterial growth (A) and surfactin concentration (B): *Bacillus subtilis* 1M (◆); *Bacillus subtilis* 3M (■); *Bacillus subtilis* 7M (▲); *Bacillus subtilis* 8M (●); *Bacillus subtilis* ATCC 21332 (●)

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 3 shows *Bacillus subtilis* 3M produced nine different surfactin isoforms and overall shows the 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 peaks 8 and 9 in the surfactin standard, which were undetected in the surfactin chromatogram of *Bacillus subtilis* 3M.



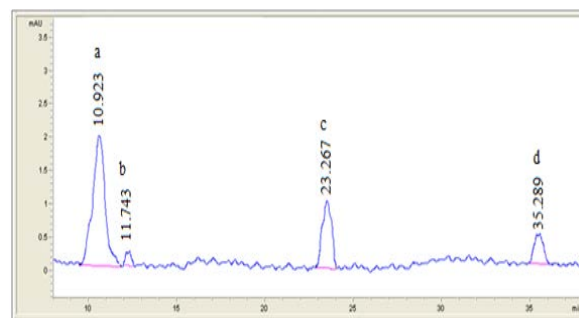
**Figure (2).** HPLC chromatogram of surfactin standard from Sigma



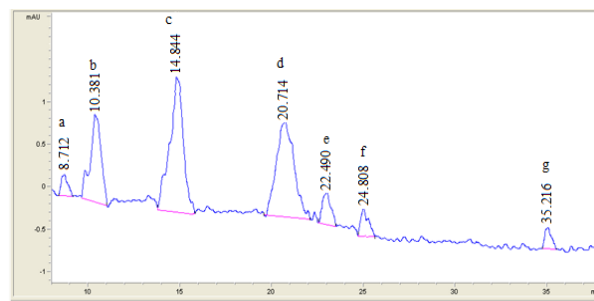
**Figure (3).** HPLC chromatogram of *Bacillus subtilis* 3M

*Bacillus subtilis* 1M produced the least number of surfactin isoforms with only four, whereas *Bacillus subtilis* ATCC 21332 as 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 dif-

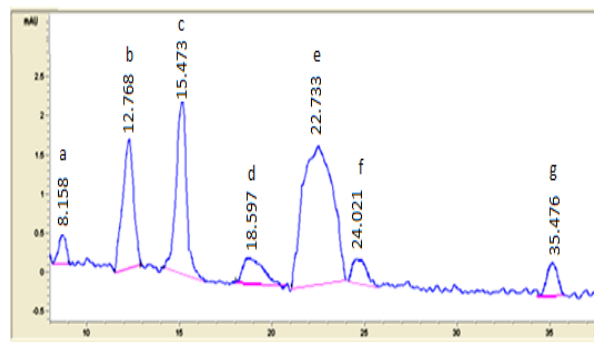
ferent isoforms were not the same in both the local isolates and *Bacillus subtilis* ATCC 21332, which may be related to strain variations. (Campos et al., 2013) stated that surfactin consists of a family of lipopeptides with similar chemical structures, i.e., isoforms, which are slightly different in 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).



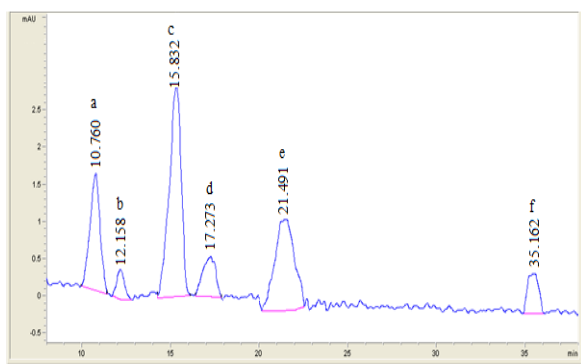
**Figure (4).** HPLC chromatogram of *Bacillus subtilis* 1M



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



**Figure (6).** HPLC chromatogram of *Bacillus subtilis* ATCC 21332



**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 30°C 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.

## ACKNOWLEDGEMENT

The authors express their gratitude towards the USIM University Malaysia lab staff and the Higher Institute of Science and Technology Al-Bayda-Libya, for providing all the valuable help.

**Duality of interest:** The authors declare that they have no duality of interest associated with this manuscript.

**Author contributions:** All listed authors in this work have worked as a team during each manuscript development stage.

**Funding:** This research did not receive any grant from funding agencies in the public, commercial, or non-profit organizations

## REFERENCES

- Abdel-Mawgoud, A. M., Aboulwafa, M. M., & Hassouna, N. A.-H. (2008). Characterization of surfactin produced by *Bacillus subtilis* isolate BS5. *Applied Biochemistry and Biotechnology*, 150, 289-303.
- Abushady, H., Bashandy, A., Aziz, N., & Ibrahim, H. (2005). Molecular characterization of *Bacillus subtilis* surfactin producing strain and the factors affecting its production. *International Journal of Agriculture and Biology*, 7(3), 337-344.
- Al-Bahry, S., Al-Wahaibi, Y., Elshafie, A., Al-Bemani, A., Joshi, S., Al-Makhmari, H., & Al-Sulaimani, H. (2013). Biosurfactant production by *Bacillus subtilis* B20 using date molasses and its possible application in enhanced oil recovery. *International Biodeterioration & Biodegradation*, 81, 141-146.
- Amit, P., & Rukhsar, A. (2013). Optimization and characterization of biosurfactant producing microbes isolated from oil contaminated soil and expression of biosurfactant genes in *E. coli*. *International Journal of Pharmaceutical Research and Bioscience*, 2(2), 46-67.
- Campos, J. M., Montenegro Stamford, T. L., Sarubbo, L. A., de Luna, J. M., Rufino, R. D., & Banat, I. M. (2013). Microbial biosurfactants as additives

- for food industries. *Biotechnology progress*, 29(5), 1097-1108.
- Chen, H.-L., Chen, Y.-S., & Juang, R.-S. (2008). Flux decline and membrane cleaning in cross-flow ultrafiltration of treated fermentation broths for surfactin recovery. *Separation and purification technology*, 62(1), 47-55.
- Cheng, Y. H., Zhang, N., Han, J. C., Chang, C. W., Hsiao, F. S. H., & Yu, Y. H. (2018). Optimization of surfactin production from *Bacillus subtilis* in fermentation and its effects on *Clostridium perfringens* - induced necrotic enteritis and growth performance in broilers. *Journal of Animal Physiology and Animal Nutrition*, 102(5), 1232-1244.
- dos Santos, S. C., Fernandez, L. G., Rossi-Alva, J. C., & de Abreu Roque, M. R. (2010). Evaluation of substrates from renewable-resources in biosurfactants production by *Pseudomonas* strains. *African journal of biotechnology*, 9(35).
- Fahle, A., Bereswill, S., & Heimesaat, M. M. (2022). Antibacterial effects of biologically active ingredients in hop provide promising options to fight infections by pathogens including multi-drug resistant bacteria. *European Journal of Microbiology and Immunology*, 12(1), 22-30.
- Isa, M. H. M., Coraglia, D. E., Frazier, R. A., & Jauregi, P. (2007). Recovery and purification of surfactin from fermentation broth by a two-step ultrafiltration process. *Journal of Membrane Science*, 296(1-2), 51-57.
- Jajor, P., Piłakowska-Pietras, D., Krasowska, A., & Łukaszewicz, M. (2016). Surfactin analogues produced by *Bacillus subtilis* strains grown on rapeseed cake. *Journal of Molecular Structure*, 1126, 141-146.
- Jiao, S., Li, X., Yu, H., Yang, H., Li, X., & Shen, Z. (2017). In situ enhancement of surfactin biosynthesis in *Bacillus subtilis* using novel artificial inducible promoters. *Biotechnology and Bioengineering*, 114(4), 832-842.
- Kinsinger, R. F., Kearns, D. B., Hale, M., & Fall, R. (2005). Genetic requirements for potassium ion-dependent colony spreading in *Bacillus subtilis*. *Journal of bacteriology*, 187(24), 8462-8469.
- Li, Y., Yang, S., & Mu, B. (2010). The surfactin and lichenysin isoforms produced by *Bacillus licheniformis* HSN 221. *Analytical letters*, 43(6), 929-940.
- Mulligan, C. N., Chow, T. Y.-K., & Gibbs, B. F. (1989). Enhanced biosurfactant production by a mutant *Bacillus subtilis* strain. *Applied microbiology and biotechnology*, 31, 486-489.
- Pereira, J. F., Gudiña, E. J., Costa, R., Vitorino, R., Teixeira, J. A., Coutinho, J. A., & Rodrigues, L. R. (2013). Optimization and characterization of biosurfactant production by *Bacillus subtilis* isolates towards microbial enhanced oil recovery applications. *Fuel*, 111, 259-268.
- Ríos, F., Fernández-Arteaga, A., Lechuga, M., & Fernández-Serrano, M. (2017). Ecotoxicological characterization of polyoxyethylene glycerol ester non-ionic surfactants and their mixtures with anionic and non-ionic surfactants. *Environmental Science and Pollution Research*, 24, 10121-10130.

- Sousa, M., Dantas, I., Feitosa, F., Alencar, A., Soares, S., Melo, V., Gonçalves, L., & Sant'ana, H. (2014). Performance of a biosurfactant produced by *Bacillus subtilis* LAMI005 on the formation of oil/biosurfactant/water emulsion: study of the phase behaviour of emulsified systems. *Brazilian Journal of Chemical Engineering*, 31, 613-623.
- Sumi, C. D., Yang, B. W., Yeo, I.-C., & Hahm, Y. T. (2015). Antimicrobial peptides of the genus *Bacillus*: a new era for antibiotics. *Canadian journal of microbiology*, 61(2), 93-103.
- Vater, J., Kablitz, B. r., Wilde, C., Franke, P., Mehta, N., & Cameotra, S. S. (2002). Matrix-assisted laser desorption ionization-time of flight mass spectrometry of lipopeptide biosurfactants in whole cells and culture filtrates of *Bacillus subtilis* C-1 isolated from petroleum sludge. *Applied and Environmental Microbiology*, 68(12), 6210-6219.
- Waewthongrak, W., Leelasuphakul, W., & McCollum, G. (2014). Cyclic lipopeptides from *Bacillus subtilis* ABS-S14 elicit defense-related gene expression in citrus fruit. *PloS one*, 9(10), e109386.
- Wei, Y.-H., & Chu, I.-M. (2002). Mn<sup>2+</sup> improves surfactin production by *Bacillus subtilis*. *Biotechnology Letters*, 24(6), 479-482.
- Wei, Y.-H., Wang, L.-F., Changy, J.-S., & Kung, S.-S. (2003). Identification of induced acidification in iron-enriched cultures of *Bacillus subtilis* during biosurfactant fermentation. *Journal of bioscience and bioengineering*, 96(2), 174-178.
- Zhi, Y., Wu, Q., & Xu, Y. (2017). Production of surfactin from waste distillers' grains by co-culture fermentation of two *Bacillus amyloliquefaciens* strains. *Bioresource Technology*, 235, 96-103.
- Zweers, J. C., Barák, I., Becher, D., Driessen, A. J., Hecker, M., Kontinen, V. P., Saller, M. J., Vavrová, L. u., & van Dijl, J. M. (2008). Towards the development of *Bacillus subtilis* as a cell factory for membrane proteins and protein complexes. *Microbial cell factories*, 7, 1-20.