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Effect of Temperature and Preservation Period on the Viability of Lyophilized *Bacillus subtilis*

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ABSTRACT: **Objective**: We sought to study the effect of temperature and preservation period on the viability of lyophilized *Bacillus subtilis*. **Methods:** Forty-five test tubes that contained suspension of *B. subtilis* ATCC 6633 were incubated for 4 hours at 37°C and washed twice before freeze-drying. Next, the lyophilized bacteria were exposed to different temperatures (-20°C, 2-8°C, and 25°C) and storage periods (30 days, 120 days, and 240 days). Further, the lyophilized bacteria were evaluated for their viability using the Miles and Misra method. Finally, the colony count of *B. subtilis* in each group was compared and analyzed using the two-way ANOVA and the Tukey-test (post-hoc test). **Results:** The lyophilized *B. subtilis* showed the viability of 93.3% (42/45) of test tubes. All of the lyophilized *B. subtilis* stored at -20°C for 30, 120, and 240 days remained viable while the bacteria stored at 2-8°C and 25°C showed the viability of 93.3% (14/15) and 86.6%, respectively. The lyophilized *B. subtilis* stored at three group temperatures and kept for 120 and 240 days showed a significant decline in their number over the control group ($p < 0.05$). **Conclusion:** In conclusion, the temperature and preservation period significantly affect the viability of the lyophilized *B. subtilis*.

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INTRODUCTION

Microculture plays a significant role in improving agricultural productivity using specific microbes such as *Bacillus subtilis*, which can improve the nutrient-absorption capacity of plant roots and enhance agriculture productivity [1-8]. At present, short- and long-term methods are used for bacterial preservation. The short-term method is done by translocating the bacteria from one growth medium to another while freeze-drying or lyophilization is exploited for the long-term method. The long-term method is usually preferred as it offers higher chances of bacterial survivability over the short-term methods. There have been reports on the effect of temperature on the viability of various lyophilized microbes [9-17]. Quite dismally, factors governing the viability of *B. subtilis* under lyophilized conditions are not well-studied. In particular, scarce is known about the optimum temperature needed for the lyophilization of *B. subtilis*. The lack of extensive research in the preservation or storage method of the microculture calls for the determination of the best method of sustaining and storing the microculture. The present study aimed to investigate the effect of temperature and preservation period on the lyophilized *B. subtilis*.

MATERIALS AND METHODS

Study outline and specimen

This is experiment-based research, which was conducted from January to September 2020 at the Microbiology Laboratory of the Atma Jaya Catholic University of Indonesia. The specimen used for this research is the rejuvenated *B. subtilis* ATCC 6633. The specimen was subjected to lyophilization under temperatures of -20°C, 2-8°C, and 25°C. This was repeated five times to ensure the consistency of the data generated.

Microbiology work-up and identification

B. subtilis was grown through an incubation period of 18 to 24 hours and subjected to re-identification. *B. subtilis* was suspended with 5 ml of sterilized NaCl 0.9%. After that, every 1 ml of the suspension was combined with 10 ml of sterilized BHI (Brain Heart Infusion) and incubated for 4 hours. Post-incubation, the suspension was centrifuged at 3000 rpm for 10 minutes. This was done to remove the precipitation and allow the mixing of the specimen with the sterilized 0.9% NaCl until it is 10 ml. Thereafter, it was subjected to the homogenization process by vortexing twice. The homogenate was then moved to a sterilized gas cylinder and subjected to lyophilization, which produced a lyophilized powder of *B. subtilis*. These specimens were kept at -20°C, 2-8°C, and 25°C for 30, 120, and 240 days of observation. The colony counting was performed using the Miles and Misra method [18]. The data generated were processed and analyzed with two-way ANOVA and the Tukey-test (post-hoc test).

RESULTS AND DISCUSSION

The preservation of *B. subtilis* at -20°C at the initial placement showed that the mean of colonies count was 3.46 × 10^3 CFU/ml. On day 30, it reduced to 2.8 × 10^3 CFU/ml while on day 120, it reduced to halved at 1.4 × 10^3 CFU/ml. Finally, on day 240, the mean was 7.8 × 10^2 CFU/ml (**Figure 1**).

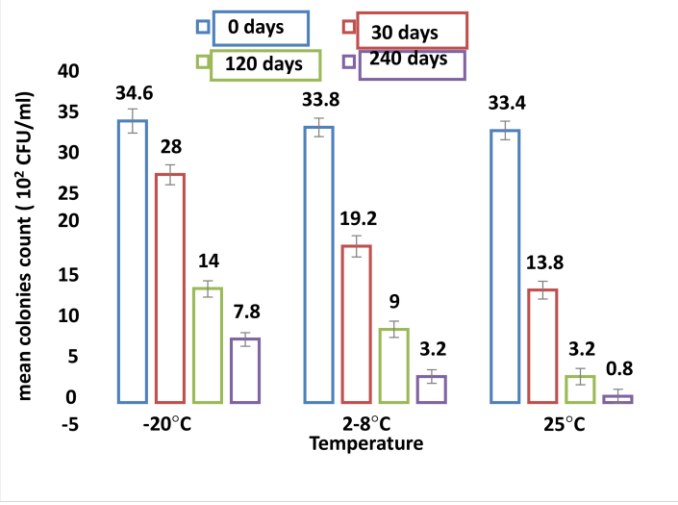

Figure 1: The mean bacterial count (CFU/ml) of lyophilized *B. subtilis* stored for 0 days (served as the control group), 30, 120, and 240 days at various temperatures (-20°C, 2-8°C, and 25°C).

The preservation of *B. subtilis* at 2-8°C at the initial placement showed that the mean of colonies count was 3.38 × 10^3 CFU/ml. On day 30, it reduced to 1.92 × 10^3 CFU/ml while on day 120, it was 9× 10^2 CFU/ml. Finally, on day 240, the mean was 3.2 × 10^2 CFU/ml.

The preservation of *B. subtilis* at 25°C at the initial placement showed that the mean of colonies count was 3.34 × 10^3 CFU/ml. On day 30, it reduced to 1.38 × 10^3 CFU/ml while on day 120, it was 3.2 × 10^2 CFU/ml. Finally, on day 240, the mean was 8 × 10^1 CFU/ml.

The two-way ANOVA revealed a significant difference between temperature and preservation period (P = 0.002). Further, the Tukey-test (post-hoc test) indicated no significant difference at initial placement and on day 30. But, a significant difference was observed on the day 120 in bacteria preserved at -20°C with 2-8°C (P = 0.015), 2-8°C with 25°C (P = 0.001), and 25°C with -20°C (P = 0.000) group. On the day 240, there was a significant difference in bacteria stored at -20°C with 2-8°C (P = 0.008), and 25°C with -20°C (P = 0.000) group. However, there was no significant difference between the 2- 8°C and 25°C temperature groups.

These findings reveal that *B. subtilis* colonies are capable of growing when stored at -20°C, 2-8°C, and 25°C for 30 and 120 days. However, different results were obtained upon storage for 240 days. In particular, the bacteria stored at -20°C showed growth while bacteria that were stored at 2-8°C had one sample that failed to grow. Finally, the bacteria stored at 25°C had two samples that showed no colony growth. Bacteria viability showed the least reduction at -20°C, followed by 2-8°C and 25°C. Our findings are in agreement with the researchers working on the lyophilization of other bacteria. For example, the lyophilized *Bifidobacterium lactis Bb-12* showed optimal viability at -18°C for 120 days, and at 7°C for 120 days. On the contrary, viability was restored for 90 days at 25°C [19]. Another study showed that the lyophilized *B. subtilis* had low viability when stored at 5°C for 10 years due to the water molecule in the ampule being activated, therefore resulting in the decline of the bacteria colony [20].

CONCLUSION

There was a significant difference in the colony count of *B. subtilis* stored at -20°C, 2-8°C, and 25°C temperatures for lyophilization. Also, *B. subtilis* stored at -20°C for 30 days, 120 days and 240 days showed better viability over 2-8°C and 25°C storage temperatures. These optimum temperatures for lyophilization that showed improved viability for *B. subtilis* must further be studied.

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AUTHOR CONTRIBUTION

KVP and ET: Conceptualization, visualization, supervision, methodology, validation, and data curation. SJ: Formal analysis, data curation, original draft preparation, review, and editing. LUK and LHM: Formal analysis and review. All authors contributed significantly to the study and have approved the final manuscript.

ETHICS STATEMENT

The authors have taken all the necessary permissions as per ethical guidelines wherever applicable. The authors will be responsible for all the technical content mentioned in the manuscript. Journal and Publisher will not be responsible for any copyright infringement and plagiarism issue.

DATA AVAILABILITY

All relevant data is present in the manuscript, and the same is accessible in the peer-review process.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

REFERENCES

1. Alori ET and Babalola OO. Microbial Inoculants for Improving Crop Quality and Human Health in Africa. *Front Microbiol* 2018;9:2213. https://doi.org/10.3389/fmicb.2018.02213
2. Trivedi P, Schenk PM, Wallenstein MD, et al. Tiny Microbes, Big Yields: enhancing food crop production with biological solutions. *Microb Biotechnol* 2017;10:999-1003. https://doi.org/10.1111/1751-7915.12804
3. Radhakrishnan R, Hashem A and Abd Allah EF. *Bacillus*: A Biological Tool for Crop Improvement through Bio-Molecular Changes in Adverse Environments. *Front Physiol* 2017;8:667. https://doi.org/10.3389/fphys.2017.00667
4. Su Y, Liu C, Fang H, et al. Bacillus subtilis: a universal cell factory for industry, agriculture, biomaterials and medicine. *Microb Cell Fact* 2020;19:173. https://doi.org/10.1186/s12934-020-01436-8
5. Hashem A, Tabassum B and Fathi Abd Allah E. Bacillus subtilis: A plant-growth promoting rhizobacterium that also impacts biotic stress. *Saudi J Biol Sci* 2019; 26(6):1291-1297. https://doi.org/10.1016/j.sjbs.2019.05.004
6. Umar A, Zafar A, Wali H, et al. Low-cost production and application of lipopeptide for bioremediation and plant growth by Bacillus subtilis SNW3. *AMB Express* 2021;11(1):165. https://doi.org/10.1186/s13568-021-01327-0
7. Naik, K., Mishra, S., Srichandan, H. et al. Microbial formulation and growth of cereals, pulses, oilseeds and vegetable crops. *Sustain Environ Res* 2020;30:10. https://doi.org/10.1186/s42834-020-00051-x
8. Hussain A, Ahmad M, Nafees M. et al. Plant-growth-promoting Bacillus and Paenibacillus species improve the nutritional status of Triticum aestivum L. *PLoS One* 2020;15(12):e0241130. https://doi.org/10.1371/journal.pone.0241130
9. Prakash O, Nimonkar Y and Shouche YS. Practice and prospects of microbial preservation. *FEMS Microbiol Lett* 2013;339(1):1-9.
10. Tan DT, Poh PE and Chin SK. Microorganism preservation by convective air-drying—A review. *Dry Technol* 2018;36:764-779. https://doi.org/10.1080/07373937.2017.1354876
11. Morgan CA, Herman N, White PA, et al. Preservation of micro-organisms by drying; A review. *J Microbiol Methods* 2006;66:183-93. https://doi.org/10.1016/j.mimet.2006.02.017
12. Kupletskaya MB and Netrusov AI. Viability of lyophilized microorganisms after 50-year storage. *Microbiology* 2011;80:850-3. https://doi.org/10.1134/S0026261711060129
13. Polo L, Mañes-Lázaro R, Olmeda I, et al. Influence of freezing temperatures prior to freeze-drying on viability of yeasts and lactic acid bacteria isolated from wine. *J Appl Microbiol* 2017;122:1603-1614. https://doi.org/10.1111/jam.13465
14. Wang GQ, Pu J, Yu XQ. et al. Influence of freezing temperature before freeze-drying on the viability of various Lactobacillus plantarum strains. *J Dairy Sci* 2020;103(4):3066-3075. https://doi.org/10.3168/jds.2019-17685
15. Bircher L, Geirnaert A, Hammes F. et al. Effect of cryopreservation and lyophilization on viability and growth of strict anaerobic human gut microbes. Microb Biotechnol 2018;11(4):721-733. https://doi.org/10.1111/1751-7915.13265
16. Manohar P and Ramesh N. Improved lyophilization conditions for long-term storage of bacteriophages. *Sci Rep* 2019;9(1):15242. https://doi.org/10.1038/s41598-019-51742-4
17. Montel Mendoza G, Pasteris SE, Otero MC. et al. Survival and beneficial properties of lactic acid bacteria from raniculture subjected to freeze-drying and storage. *J Appl Microbiol* 2014;116(1):157-66. https://doi.org/10.1111/jam.12359
18. Miles AA, Misra SS and Irwin JO. The estimation of the bactericidal power of the blood. *J Hyg (Lond)* 1938;38:732-49. https://doi.org/10.1017/s002217240001158x
19. Da Silva TM, Lopes EJ, Codevilla EJ, et al. Development and characterization of microcapsules containing *Bifidobacterium* Bb-12 produced by complex coacervation followed by freeze drying. *LWT-Food Sci Technol* 2018; 90:421-417. https://doi.org/10.1016/j.lwt.2017.12.057
20. Miyamoto-Shinohara Y, Sukenobe J, Imaizumi T, et al. Survival of freeze-dried bacteria. *J Gen Appl Microbiol* 2008;54:9-24. https://doi.org/10.2323/jgam.54.9