Journal of Clinical and Nursing Research, 2024, Volume 8, Issue 7
http://ojs.bbwpublisher.com/index.php/JCNR
Online ISSN: 2208-3693
Print ISSN: 2208-3685

Isolation and Experimental Study on Bacteriostasis of *Bacillus haynesii*

Jiedan Liao[1], Mengbo Yu[2,3], Jiaming Li[2], Zhiheng Xie[2], Wanjun Gu[1,2], Shuzhen Zhou[2*], Yi Ma[3*]

[1]College of Animal Science and Technology, Foshan University, Foshan 528225, Guangdong Province, China
[2]Foshan City Nanhai Eastern Along Pharmaceutical Co., Ltd, Foshan 528234, Guangdong Province, China
[3]Department of Veterinary Medicine, College of Coastal Agricultural Sciences, Guangdong Ocean University, Zhanjiang 524088, Guangdong Province, China

***Corresponding authors:** Shuzhen Zhou, zhousz@easternalong.com; Yi Ma, mayi761@163.com

Copyright:© 2024 Author(s). This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY 4.0), permitting distribution and reproduction in any medium, provided the original work is cited.

Abstract: This experiment aims to isolate and inhibit three bacteria strains to provide candidate strains for the development and application of probiotics. Using bacterial morphological identification, 16S rDNA sequence alignment, and genetic evolution analysis, three strains were identified as *Bacillus haynesii*, named HP01, HD02, and HK03. Through biosurfactant activity tests, C-TAB tests, hemolysis tests, and antibacterial activity analyses, the results showed that all three strains of *B. haynesii* exhibited significant biosurfactant activity. Additionally, the solutions of the three strains demonstrated a pronounced antibacterial effect on *Staphylococcus aureus*. The resistance and safety of commonly used drugs were evaluated using the tablet diffusion method and a mouse feeding test. The results indicated that the three strains were not resistant to commonly used antibacterial drugs, and the oral bacterial solution was not pathogenic and had high safety in mice. The study concluded that all three *B. haynesii* strains met the basic conditions for use, with *B. haynesii* HP01 being the most promising candidate.

Keywords: *Bacillus haynesii*; Separation identification; Antibacterial activity; Safety

Online publication: August 13, 2024

1. Introduction

Bacillus haynesii is a new species of *Bacillus* identified in 2017, and its representative strain is NRRL B-41327T [1]. Since the confirmation of this new species, researchers have conducted in-depth studies on its natural and biological engineering environment, bioenzyme engineering, biofertilizer production, and biofilm formation [2,3]. Abdelgalil *et al.* aimed to expand the acid phosphatase production and biolytic capacity of phosphate ore using the newly discovered *B. haynesii* [4]. Marín-Sanhueza *et al.* studied the formation of different stress response molecules by CamB6, isolated from a hot spring in Chile, and characterized polymers produced by this strain using different physicochemical techniques [5]. Wang *et al.* reported the synthesis of stable copper nanoparticles (NPs) using cheap and non-toxic copper salts in an aqueous solution [6]. NPs were obtained by phylogenetic analysis, revealing that NPs form in cell-free culture medium but not in fresh biomass medium, suggesting the involvement of an extracellular compound in this process. In this experiment, three *B.*

haynesii strains were purified, Gram-stained, and subjected to 16S rDNA sequence alignment. The biosurfactant characteristics produced by the strains were compared and analyzed through antibacterial activity tests to provide candidate strains for the development and application of *B. haynesii* probiotics.

2. Materials and methods

2.1. Materials

LB liquid medium, LB agar medium, cecetyltrimethylammonium bromide agar medium (CTAB), MRS liquid medium, and blood agar medium were purchased from Guangdong Huankai Microbiology Technology Co., LTD. Indicators, *Escherichia coli* (K12D31) and *Staphylococcus aureus* (ATCC 29213), were purchased from the China Industrial Microbial Seed Conservation and Management Center. BALB/c mice were purchased from the Guangdong Laboratory Animal Center. Drug sensitivity paper was purchased from Thermo Fisher (China) Technology Co., Ltd. Guangzhou Eki Biotechnology Co., Ltd. was responsible for the 16S rDNA sequencing.

2.2. Methods

2.2.1. Indicator bacteria

Staphylococcus aureus and *E. coli* were inoculated in LB medium at 37°C and shaken at 120 r/min for about 16 to 24 hours, then stored at 4°C [7].

2.2.2. Isolate the bacteria for culture

The isolated strains were cultured on LB plain agar plates. Typical colonies were picked and inoculated in MRS medium, then shaken at 120 r/min for 24 hours at 37°C. The bacterial solution was then stored at 4°C.

2.2.3. Morphological identification

Three isolates were inoculated on LB plain agar plates and cultured at 37°C for 24 hours to observe the colony morphology. The body morphology and staining characteristics were observed after Gram staining.

2.2.4. 16S rDNA analysis of genetic evolution

The 16S rDNA gene fragments of purified cultures of the three isolates were sequenced and compared to the 16S rDNA genetic development tree. The probiotic fermentation product isolate was named *B. haynesii* HP01, the turtle pond sediment isolate was named *B. haynesii* HD02, and the Xianxi Lake sediment isolate was named *B. haynesii* HK03.

2.2.5. Biosurfactant detection

(1) Emulsification activity (EA): Take 2.5 mL of a 24-hour culture, centrifuge at 10,000 r/min, draw 2 mL into a centrifuge tube, vortex the mixture for 2 minutes, divide the height of the emulsion layer by the total height of the mixture, and multiply by 100 to measure and calculate the emulsification capacity.

(2) Emulsification index detection (E24): Place the emulsification activity detection tube at 4°C for 24 hours, divide the measured height of the emulsion layer by the total height of the mixture, and multiply by 100 to calculate E24.

2.2.6. Hemolysis test

10 µL of bacterial solution was added to the filter paper affixed to the blood agar medium and cultured at 37°C

for 24 hours to observe the moss or hemolytic halo, determining the intensity of biosurfactant activity. A 20% Tween-80 solution served as a positive control.

2.2.7. CTAB trial

Using CTAB agar medium, according to **Section 2.2.6.**, 10 μL of bacterial liquid was added. A 20% Tween-80 solution served as a positive control. The cultures were incubated at 37°C for 24 hours and then stored at 4°C to detect anionic biosurfactant. Judgment criteria: a dark blue halo around the CTAB agar plate indicated a positive result, while no dark blue halo indicated a negative result.

2.2.8. Drug sensitive test

The antibiotic sensitivity of the three isolates to 14 commonly used drugs, including Amoxicillin, Amikacin, Gentamicin, Enrofloxacin, Ciprofloxacin, Spectinomycin, Cefotaxime, Florfenicol, Veomycin, Ceftriaxone, Doxycycline, Penicillin, Cephalosporin, and Ofloxacin, was determined by the paper diffusion method.

2.2.9. Bactericidal test

6 mm diameter filter sheets were autoclaved at 121°C for 20 minutes and stored in a 60°C drying box. 10 μL of bacterial solution was placed on the surface of the indicator plate filter paper. *E. coli* indicator plate (blank control: neomycin) and *S. aureus* indicator plate (blank control: culture medium) were incubated at 37°C, and the antibacterial zone diameter was measured after 24 hours.

2.2.10. Safety test

After 2 days of feeding, the mice were randomly divided into an experimental group (3 groups) and a control group (1 group), with 7 mice per group. On the first day, the experimental group received 0.1 mL of bacterial solution. From the next day, each mouse received 0.1 mL/day added to their drinking water for 7 days. The mice's mental status, diet, stool condition, morbidity, and mortality were observed daily. Test and control mice were weighed on days 3 and 7, and the results were recorded.

3. Results

3.1. Morphological identification

The three strains were positive for Gram stain. HP01 and HD03 are larger bacteria that form endospores, with the bud in or near the bacterial body, appearing as long ellipses or rods. HD02 is slender in the center of the bacterial body. The results are shown in **Figure 1**.

The colony morphology results of the three strains after 37°C culture showed that HP01 and HD02 colonies were light milky yellow, round, and about 3–5 mm in diameter. These colonies are flat, dry, radial, and translucent with a distinct periphery. The colonies of the HK03 strain are milky white, round, about 1–2 mm in diameter, and transparent around the edges, making it difficult to form independent colonies. The results are shown in **Figure 2**.

3.2. 16S rDNA analysis of genetic evolution

The 16S rDNA gene fragments were sequenced from the three isolates and compared with the GenBank database to establish a genetic evolution analysis. The results are shown in **Figure 3**. Strains HP01, HD02, and HK03 share 86% identity with *B. haynesii* NRRL B-41327T and were assigned to *B. haynesii*.

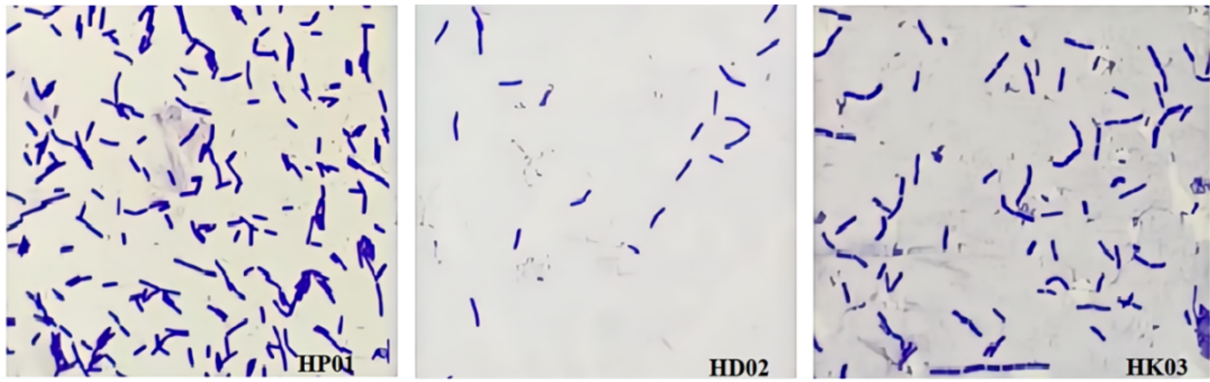

Figure 1. Gram stain plots of the isolates

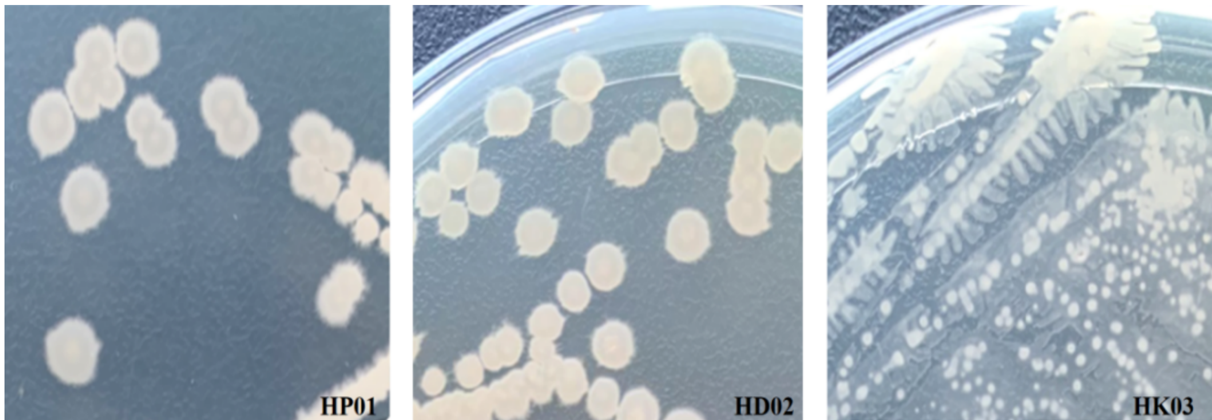

Figure 2. Bacterial colony plots of the isolates

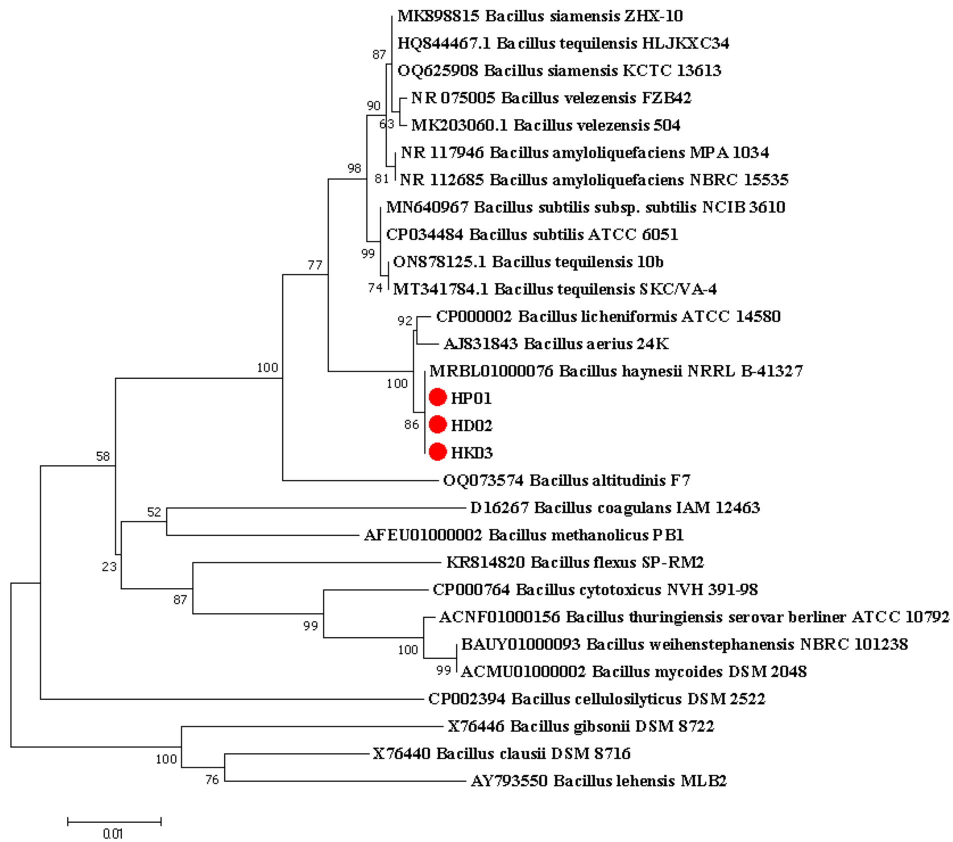

Figure 3. Strains of *Bacillus haynesii* genetic tree

3.3. Emulsification activity test

The emulsification index of *B. haynesii* is as follows: 58% for HP01, 55% for HD02, and 60% for HK03. The biological surface activity of the three strains, from strongest to weakest, is *B. haynesii* HK03 strain, HP01 strain, and HD02 strain. The emulsification index is 43% for HP01, 38% for HD02, and 45% for HK03. The results are shown in **Figure 4**.

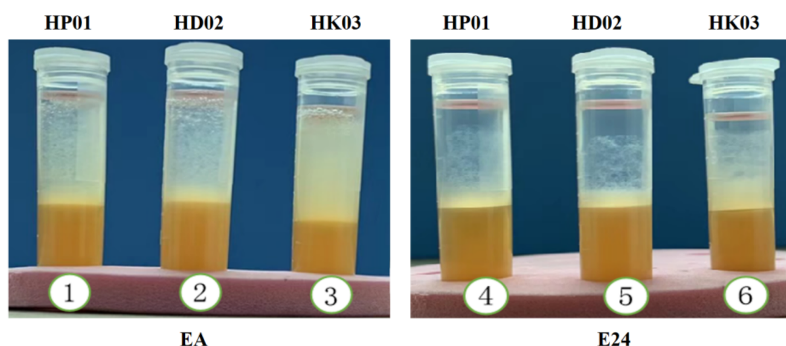

Figure 4. Emulsification activity test of the isolates

3.4. CTAB test

The results of the CTAB agar test for *B. haynesii* strains are shown in **Figure 5**, displaying blue halos of varying intensities around the colonies, with the HP01 strain being the most prominent. The number seven is a 20% Tween 80 positive control.

3.5. Hemolysis Test

Three strains formed on blood agar plates, including the HP01 strain. The results of the hemolysis test are shown in **Figure 6**. The number seven is a 20% Tween 80 positive control.

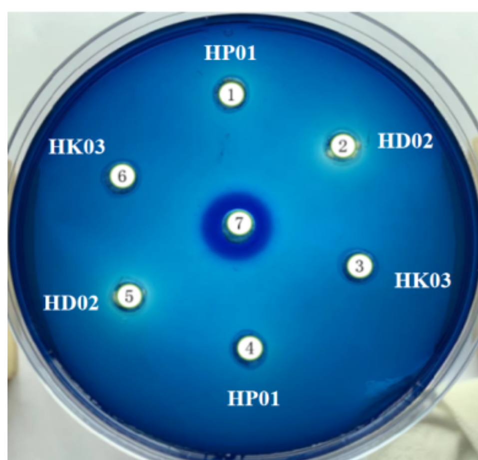

Figure 5. CTAB test of the isolates

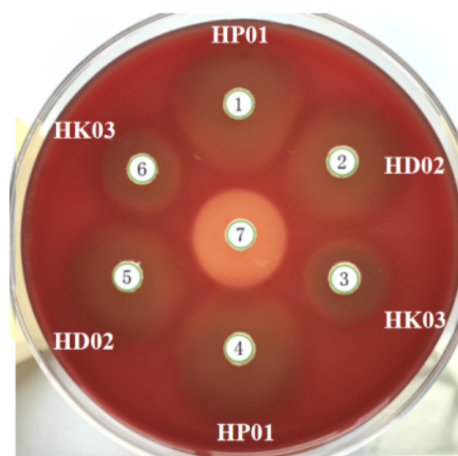

Figure 6. Hemolysis test of the isolates

3.6. Drug sensitivity test

The results of drug susceptibility testing of the three isolates with 14 common antimicrobial agents are shown in **Table 1**. A bacteriosphere diameter below 10 mm indicates insensitivity or low sensitivity, while a diameter

over 15 mm indicates high or extreme sensitivity. The results showed that the three *B. haynesii* strains were more susceptible to most of the drugs, indicating that they were not resistant.

Table 1. Results of drug sensitivity test (bacteriosphere diameter in mm)

Drugs	*Bacillus haynesii* HP01	*Bacillus haynesii* HD02	*Bacillus haynesii* HK03
Amoxicillin	22	24	25
Amikacin	21	24	24
Gentamicin	20	20	20
Enrofloxacin	25	26	24
Ciprofloxacin	23	25	24
Spectinomycin	18	19	19
Cefotaxime	28	26	26
Florfenicol	26	27	27
Neomycin	18	17	20
Ceftriaxone	22	24	25
Doxycycline	26	25	24
Penicillin	14	15	24
Cephalosporin	22	22	23
Ofloxacin	25	26	28

3.7. Bactericidal test

Using *S. aureus* and *E. coli* as indicator bacteria, the results showed that the coil diameters of strains HP01, HP02, and HP03 are 13 mm, 12 mm, and 11 mm, respectively. For *E. coli*, the coil diameters are 9 mm, 8 mm, and 8 mm, respectively. It can be seen that, compared to *S. aureus*, the strains have weaker antibacterial activity against *E. coli*, with the antibacterial intensity as follows: HP01 > HD02 > HK03. The results of the antibacterial test are shown in **Figure 7**. The bacterial fluids of the three isolates inhibited both *S. aureus* and *E. coli*, showing an obvious antibacterial effect on *S. aureus*.

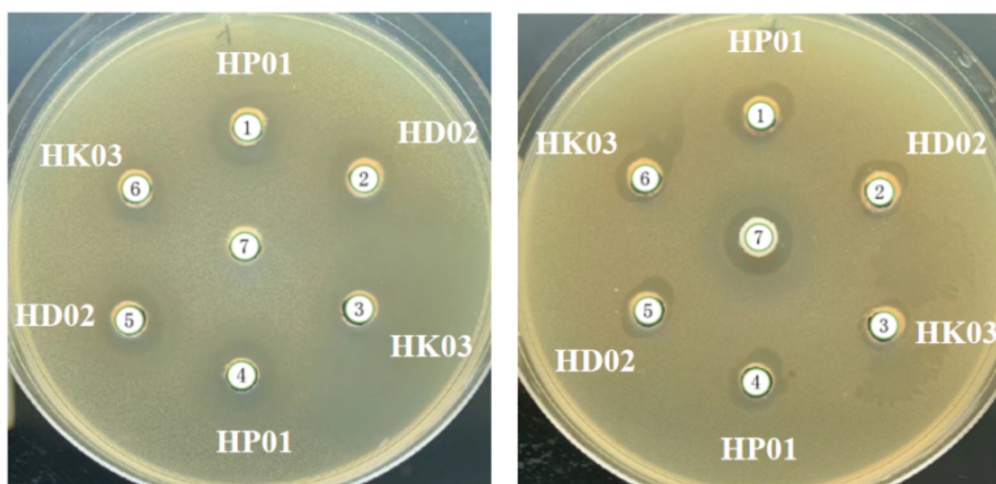

Figure 7. Bactericidal test. *Staphylococcus aureus* (ATC 29213) was used as the indicator bacteria on the left plate, while *Escherichia coli* (K12D31) was used as the indicator bacteria on the right plate. Number 7 is a blank control, where the left is the culture medium, while the right is neomycin

3.8. Safety test

In the feeding test, the mice were in good mental state, had a normal diet, normal feces, and had no morbidity or death. There was no difference in weight gain between the test group and the control group during the test period, indicating no pathogenic or toxic side effects.

4. Discussion

4.1. Biological surface activity

This laboratory isolated three strains from Xianxi Lake sediment and focused on surfactant production, marking the first report in China. Palit and Das revealed the cellulolytic bacterial diversity in the mangrove ecosystem and elucidated the cellulose degradation mechanism of *B. haynesii* DS7010 under pH, salinity, and lead-modified conditions [8]. The abundance of cellulose-degrading heterotrophic bacteria in mangrove sediments was higher than in water. The most promising strain, *B. haynesii* DS7010, exhibited endoglucanase, exoglucanase, and β-glucosidase activity, with the greatest degradation at 48 hours of incubation and 1% substrate concentration at 41°C. These results demonstrate the cellulose degradation mechanism of mangrove bacteria and their potential for environmental recovery in response to pollution and climate change. The three *B. haynesii* strains isolated and identified in this study all exhibit varying degrees of surface activity. Further research is needed to determine whether they produce related enzymes and if they are beneficial for environmental treatment.

4.2. Antibacterial study

Koşarsoy Ağçeli showed that levan is a biopolymer with many different uses, and temperature is an important parameter in its synthesis [9]. Some studies reported the production of levan by *B. haynesii* in the temperature range from 4°C to 95°C, with results showing that levan production at 37°C was 10.9 g/L. Among the samples synthesized at 4°C, the highest emulsion volume was 83.4%. The average antioxidant activity of all levan samples synthesized at different temperatures was 84%. All synthetic levan samples exhibited bacteriostatic effects against pathogenic bacteria. Furthermore, levan synthesized at 45°C showed the highest antibacterial efficacy against *E. coli* ATCC 35218, with an inhibitory zone of 21.3 ± 1.82 mm. From the perspective of candidate animal probiotics, the three *B. haynesii* strains all have basic requirements, especially the *B. haynesii* HP01 strain.

4.3. Resistance and safety studies

Bacillus is a probiotic candidate without pathogenic and toxic side effects. Rahman *et al.* isolated 36 species from marine soil bacteria and performed *in vitro* screening to determine their antibacterial activity against fish pathogens [10]. Two types of antagonistic bacteria, *B. haynesii* CD223 and *Advenella mimigardefordensis* SM421, were identified. These two bacteria were added to fish diets and fed to Nile tilapia for 90 days. The results showed that the strains could significantly promote fish growth and improve hematological parameters and IgM levels. By adding these bacteria to fish feed, they regulated the intestinal flora and reduced the load of pathogenic enterococci, providing a disease-prevention effect in Nile tilapia. In this experimental study, a susceptibility test of 14 antimicrobial drugs was conducted, and the results showed that the three *B. haynesii* strains were sensitive to most drugs and were not resistant. The safety feeding test results showed that the mice had a good mental state, normal diet, normal feces, and no morbidity or death. There was no difference in weight gain between the test group and the control group during the test period, indicating no pathogenic

effects or toxic side effects. The drug susceptibility test and the mouse safety feeding test further established the application potential of the three *B. haynesii* strains as candidate species for animal probiotics.

Funding

(1) Natural Science Foundation of Guangdong Province in 2023 (No. 2023A1515012181)
(2) Self-funded Science and Technology Innovation Project of Foshan City in 2022 (No. 220001005797)
(3) Basic and Applied Basic Research Foundation of Guangdong Province in 2022 (No. 2022A1515140052)
(4) Innovation Project of Guangdong Graduate Education in 2022 (No. 2022JGXM129, No. 2022JGXM128) and 2023 (No. 2023ANLK-080)

Disclosure statement

The author declares no conflict of interest.

References

[1] Dunlap CA, Schisler DA, Perry EB, et al., 2017, Bacillus swezeyi sp. nov. and Bacillus haynesii sp. nov., Isolated from Desert Soil. Int J Syst Evol Microbiol, 67(8): 2720–2725. https://doi.org/10.1099/ijsem.0.002007

[2] Rajitha K, Nancharaiah YV, Venugopalan VP, 2020, Acid Soluble Extracellular Matrix Confers Structural Stability to Marine Bacillus haynesii Pellicle Biofilms. Colloids and Surfaces B: Biointerfaces, 194: 111160. https://doi.org/10.1016/j.colsurfb.2020.111160

[3] Maurya A, Kumar R, Yadav P, et al., 2022, Biofilm Formation and Extracellular Polymeric Substance (EPS) Production by Bacillus haynesii and Influence of Hexavalent Chromium. Bioresour Technol, 352: 127109. https://doi.org/10.1016/j.biortech.2022.127109

[4] Abdelgalil SA, Kaddah MMY, Duab MEA, et al., 2022, A Sustainable and Effective Bioprocessing Approach for Improvement of Acid Phosphatase Production and Rock Phosphate Solubilization by Bacillus haynesii Strain ACP1. Sci Rep, 12(1): 8926. https://doi.org/10.1038/s41598-022-11448-6

[5] Marín-Sanhueza C, Echeverría-Vega A, Gómez A, et al., 2022, Stress Dependent Biofilm Formation and Bioactive Melanin Pigment Production by a Thermophilic Bacillus Species from Chilean Hot Spring. Polymers (Basel), 14(4): 680. https://doi.org/10.3390/polym14040680

[6] Wang W, Zhu L, Lv P, et al., 2018, Novel Candy-like Cu4O3 Microstructure: Facile Wet Chemical Synthesis, Formation Mechanism, and Good Long-Term Antibacterial Activities. ACS Appl Mater Interfaces, 10(43): 37287–37297. https://doi.org/10.1021/acsami.8b14470

[7] Zhou S, Gu Z, Zhang R, et al., 2023, Identification and Antimicrobial Test of Bacillus velezensis. Guangdong Journal of Animal and Veterinary Science, 48(4): 30–34 + 41.

[8] Palit K, Das S, 2024, Cellulolytic Potential of Mangrove Bacteria Bacillus haynesii DS7010 and the Effect of Anthropogenic and Environmental Stressors on Bacterial Survivability and Cellulose Metabolism. Environ Res, 252(Pt 1): 118774. https://doi.org/10.1016/j.envres.2024.118774

[9] Koşarsoy Ağçeli G, 2023, Similarities and Differences of Nano-Sized Levan Synthesized by Bacillus haynesii at Low and High Temperatures: Characterization and Bioactivity. Int J Biol Macromol, 253(Pt 3): 126804. https://doi.org/10.1016/j.ijbiomac.2023.126804

[10] Rahman MM, Paul SI, Rahman A, et al., 2022, Suppression of Streptococcosis and Modulation of the Gut Bacteriome in Nile Tilapia (Oreochromis niloticus) by the Marine Sediment Bacteria Bacillus haynesii and Advenella

mimigardefordensis. Microbiol Spectr, 10(6): e0254222. https://doi.org/10.1128/spectrum.02542-22

Publisher's note

Bio-Byword Scientific Publishing remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.