MIJRD Multidisciplinary International Journal of Research and Development

Volume: 02 / Issue: 03 / 2023 - Open Access - Website: www.mijrd.com - ISSN: 2583-0406

Biological Activities of Bacterial Isolates from Adiangao Cave, San Jose Camarines Sur

Liezel DC. Atole

College of Arts and Science, Partido State University, Goa, Camarines Sur

Abstract— Considered an extreme environment, caves possessed a unique and poorly studied ecosystem comprising a unique microbial community carrying out various biological activities. This study aimed to isolate, identify, and characterize the biological activities of bacteria isolated from soil samples in Adiangao Cave, San Jose, Camarines Sur. Seventeen bacterial isolates were identified and characterized in terms of enzymatic activity, phosphate solubilizing activity, formaldehyde resistance, and antagonistic activity. Based on 16S rDNA sequencing, the most prevalent isolates obtained were members of the Bacillus genera. Results have shown that 11 isolates exhibited amylase activity, and 12 had cellulase activity, showing its potential for industrial application. Eight isolates were positive for phosphate solubilization, which can be used for plant growth promotion and heavy metal immobilization. It was also shown that 13 isolates could grow under the presence of formaldehyde showing its potential application for the bioremediation of formaldehyde. Isolate CS31, identified as Bacillus paranthracis, is noted to have great potential for the production of antimicrobial compounds that can inhibit pathogenic microorganisms such as Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, and Salmonella typhimurium. The results of this study demonstrated the importance of cave bacteria for the production of valuable bioactive compounds that are commercially important.

Keywords— Adiangao Cave, biological activity, antimicrobial.

I. INTRODUCTION

One of the unique and poorly studied ecosystems on Earth is caves. Considered an extreme environment, caves generally have a low and stable temperature, minimum light, low nutrients, and high humidity (Palmer 1991; Tomczyk-Żak and Zielenkiewicz 2015). The most common caves are karstic caves that are formed through geomorphological and microbiological processes (Engel 2010; Tisato et al., 2015; Bontognali et al., 2016).

Studies have shown that various microorganisms can be isolated from various caves worldwide, including Gram-positive bacteria, Gram-negative bacteria, protozoa, and fungi (Gulecal-Pektas, 2016, Yasir, 2018). Due to their unique environmental conditions, caves are composed of unique microbial communities.

Some microbial isolates were identified to be causing rock weathering processes and biomineralization, while some are carrying out various enzymatic activities (Bindschedler S, Cailleau G, Verrecchia E. Role of Fungi in the Biomineralization of Calcite. Minerals. 2016; 6(2):41. https://doi.org/10.3390/min6020041).

These microorganisms are thriving in unfavorable growth conditions with many limiting factors. With the combinations of these different unique conditions, it is expected to harbor novel microorganisms producing unique beneficial chemical compounds with biotechnological benefits.

Volume: 02 / Issue: 03 / 2023 - Open Access - Website: www.mijrd.com - ISSN: 2583-0406

One of the identified caves in San Jose Camarines Sur is the Adiangao Cave, located at Barangay Adiangao, San Jose, Camarines Sur. It is popular with locals due to its chain of grottoes, and the numerous stalactites and stalagmites that can be seen along the floor and ceiling of the caves. Bacterial diversity and their biological activities have not yet been studied, hence, this study aims to isolate, identify, and characterize the biological activities of bacteria isolated from soil samples of Adiangao Cave.

II. PROCEDURE FOR PAPER SUBMISSION

A. Isolation and identification of bacterial isolates from soil samples.

Soil samples were collected from Adiangao Cave located in Adiangao, San Jose, Camarines Sur (13°43.376' N, 123°41.255' E) and were transported in a cooler and immediately processed in the laboratory. Samples were serially diluted using 9ml of 0.1% (w/v) peptone water tubes. Diluted samples were then plated on Nutrient Agar (NA) and were incubated at 37°C for 24 h. Colonies were enumerated, and counts were expressed in log10 CFU/ml.

B. Molecular identification of bacterial isolates

One ml of 24 h culture was centrifuged at 4,500 rpm for 5 min. The supernatant was discarded, and the pellet was resuspended in 1 ml of sterile double distilled water. The suspensions were then sent to Kinovett for molecular identification. Macrogen Inc., in South Korea, did the sequencing of 16S rDNA PCR amplicons of the isolates. Partial 16S rDNA sequences were imported, checked for sequencing quality, and trimmed using the Staden Package software (Staden, 1996) after which the consensus sequences were compiled and matched to the reference strains found in the National Center of Biotechnology Information (NCBI) GenBank database through BLASTn (Basic Local Alignment Search Tool) (NCB1, 2013).

C. Biological activities of bacterial isolates

1. Screening for Cellulase and Amylase Activity

Qualitative analysis for cellulase and amylase activity was based on Kim et al., (2014) with minor modifications for amylase activity i.e., instead of carboxymethylcellulose (CMC), 1% soluble starch was used as the sole carbon source, respectively. Pure isolates were point-inoculated on Minimal Salts Agar plates (containing 0.5% CMC, 0.1% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.1% KCl, 0.05% yeast extract, 1.5% agar for cellulose agar plates; and 0.3% beef extract, 1% soluble starch and 1.2% agar for starch agar plates) and incubated at 37°C for 48 h. Following incubation, plates for cellulase activity detection were flooded with 1% Congo red for 15 min. The Congo red solution was then poured off, and plates were further flooded with 1 M NaCl for 15 min. Colonies exhibiting zones of the clearing were taken as positive cellulose-degrading bacterial colonies. Plates for detecting amylase activity were flooded with Gram's iodine for 3 to 5 min and zones of the clearing were observed.

2. Screening for phosphate-solubilizing bacteria

Plate assay was done to determine the ability of the bacterial isolates to solubilize phosphate using Pikovskaya agar. Isolates were point-inoculated in the medium and were incubated for 48 hours at 37°C. After incubation, zones of clearing around colony growth were observed for phosphate solubilizing isolates.

Screening for the formaldehyde-resistant bacterium

Volume: 02 / Issue: 03 / 2023 - Open Access - Website: www.mijrd.com - ISSN: 2583-0406

Growth of the bacterial isolates was then tested in a modified DM-3 medium (30 g of NaCl, 5 g tryptone, 5 g of yeast extract, 6 g of Na2HPO4, 3 g of KH2PO4, 1 g of NH4Cl, 1.2 g of MgSO4, and 111 mg of CaCl2 in 1 L) with 1% formaldehyde concentration. Plates were incubated at 37°C for 48 h. After incubation, bacterial growth was then assessed.

3. Antagonistic activity against pathogenic microorganisms

Agar well diffusion assay was used to test the ability of bacterial isolates to inhibit pathogenic microorganisms, i.e., E. coli P. aeruginosa, S. aureus, B. cereus, and S. typhimurium. Test organisms were swabbed into Mueller Hinton Agar, MHA, (Sigma-Aldrich) and 5 mm wells were bored on the agar. Bacterial isolates were also grown in Nutrient Broth, NB, and incubated for 24 hours at 37°C. After incubation, cultures were centrifuged at 12,000 rpm for 3 min, and the supernatants were collected. Then, 100 µL of supernatants were added to the respective wells. Plates were incubated for 24 hours at 37°C. Cefuroxime served as the positive control, and sterile distilled water was used as the negative control. Antimicrobial activity was detected by measuring the zone of inhibition that appeared after the incubation period.

III. RESULTS AND FINDINGS

Sampling and Characterization of bacterial isolates from Adiangao Cave

Soil samples were collected at the dark zone of Adiangao Cave (Figure 1). From the cultured plates, seventeen bacterial isolates were obtained and purified using NA.

Figure 1. Adiangao Cave located at Barangay Adiangao, San Jose, Camarines Sur.

Identification of Bacterial Isolates

All purified isolates were identified based on the 16S rDNA sequence. The obtained consensus sequence of each isolate was matched to the reference strains found in the National Center of Biotechnology Information (NCBI) GenBank database through BLASTn (Basic Local Alignment Search Tool). Analysis of the identified isolates showed that the majority of the isolates were members of the Bacillus genera as presented in Table 2.

MIJRD Multidisciplinary International Journal of Research and Development

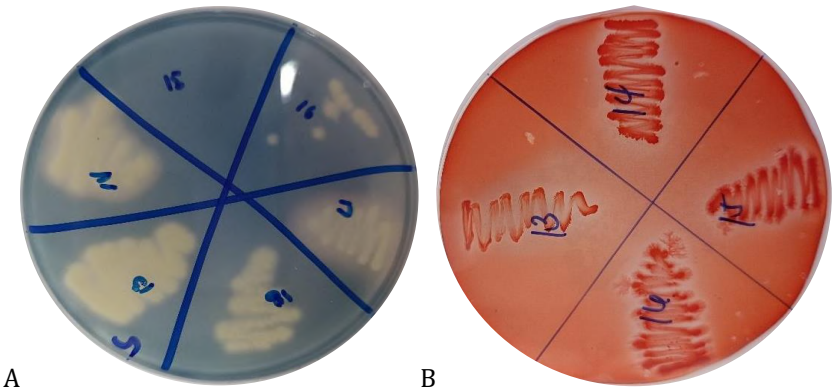
Volume: 02 / Issue: 03 / 2023 - Open Access - Website: www.mijrd.com - ISSN: 2583-0406

Table 2. Genbank accession number, % identity, and species identity of bacterial isolates from cave soil samples.

Isolate	Genbank Accession Number	Identity (%)	Species
CS41	ON005108.1	98.98	Bacillus cereus
CS34	CP054831.1	99.51	Staphylococcus saprophyticus
CS2	MZ950778.1	98.58	Bacillus pseudomycoides
RC2	KT900621.1	99.54	Bacillus sp.
G3	ON005108.1	98.98	Bacillus cereus
G2	ON012959.1	100	Bacillus subtilis
CC1	MW012643.1	98.39	Bacillus subtilis
LC1	ON000579.1	99.76	Bacillus tropicus
SM1	ON000579.1	99.76	Bacillus tropicus
G1	ON000579.1	99.76	Bacillus tropicus
CC4	KX946197.1	81.50	Bacillus licheniformis
LC5	ON012959.1	99.89	Bacillus subtilis
RC4	OP903925.1	96.22	Bacillus cereus
CS31	MK508861.1	99.86	Bacillus paranthracis
CS33	OP592255.1	99.86	Bacillus paranthracis
CS42	MN938501.1	99.86	Bacillus inaquosorum
CS52	MW405872.1	99.71	Bacillus atrophaeus
RC2	KY074395.1	100	Bacillus cereus

Biological activities of bacterial isolates

Different biological activities of each isolate were determined to further understand its possible importance for industrial application. Each isolate was screened for its ability to produce enzymes responsible for biomass degradation. The activity of these isolates were summarized in Table 3.



A B

Figure 2. Plate assay screening of cave bacterial isolates: (a) amylase, (b) cellulase. A zone of clearing around bacterial growth indicates a positive result for enzymatic activity.

Volume: 02 / Issue: 03 / 2023 - Open Access - Website: www.mijrd.com - ISSN: 2583-0406

Results showed that 11 out of 17 isolates exhibited amylase activity, and 12 had cellulase activity. Based on these results, it was confirmed that bacterial isolates from cave samples possess enzymatic activities and can be explored for possible industrial use. Bacterial isolates were also screened for their ability to solubilize phosphate. Results have shown that 8 isolates were positive for phosphate solubilization that can be used for plant growth promotion and heavy metal immobilization. The ability of the isolates to grow in a formaldehyde-containing medium was also determined, and results showed that 13 isolates out of 17 were still capable of growing under the presence of formaldehyde. This shows that the isolates can use formaldehyde as a carbon source and has the potential application for the bioremediation of formaldehyde.

Table 3. Biological activities of bacterial isolates isolated from cave soil samples.

Code	Plate Assays			
	Amylase Plate Assay	Cellulase Plate Assay	Pikovskaya Plate Assay	Growth in Formaldehyde Medium
CS33	+	-	-	+
CS41	-	+	-	-
CS34	-	+	+	+
CS52	-	+	-	+
CS42	+	-	-	+
CS31	+	+	-	-
CS2	+	-	+	-
RC2	-	+	-	+
G3	-	+	+	+
G2	+	+	+	+
CC1	+	+	-	+
LC1	-	-	+	+
SM1	+	+	+	+
G1	+	+	-	+
CC4	+	+	+	+
LC5	+	+	-	-
RC4	+	-	+	+

Many of the Bacillus genera are capable of producing these extracellular enzymes that target various macromolecules such as carbohydrates, lignin, organic phosphate, proteins, and sugars (Harirchi et al., 2022). Some of these extracellular enzymes are being used by microorganisms to overcome competition with other microorganisms. Enzymes such as cellulases and amylases are known for their industrial importance. Amylases from Bacillus sp. have many applications in the food, fermentation, textile, and paper industries (de Silveria et al., 2019).

Volume: 02 / Issue: 03 / 2023 - Open Access - Website: www.mijrd.com - ISSN: 2583-0406

Antimicrobial activity against pathogenic microorganism

Isolated bacterial isolates were tested for antimicrobial activity against E. coli, P. aeruginosa, S. aureus, B. cereus, and S. typhimurium. Results have shown that isolate CS31, Bacillus paranthracis, demonstrated a significant inhibitory effect against P. aeruginosa, E. coli, S. aureus, and S. typhimurium. On the other hand, isolate G2, Bacillus subtilis, showed the highest inhibitory effect against B. cereus. Based on statistical analysis, the activity of these promising isolates was significantly different compared to other isolates.

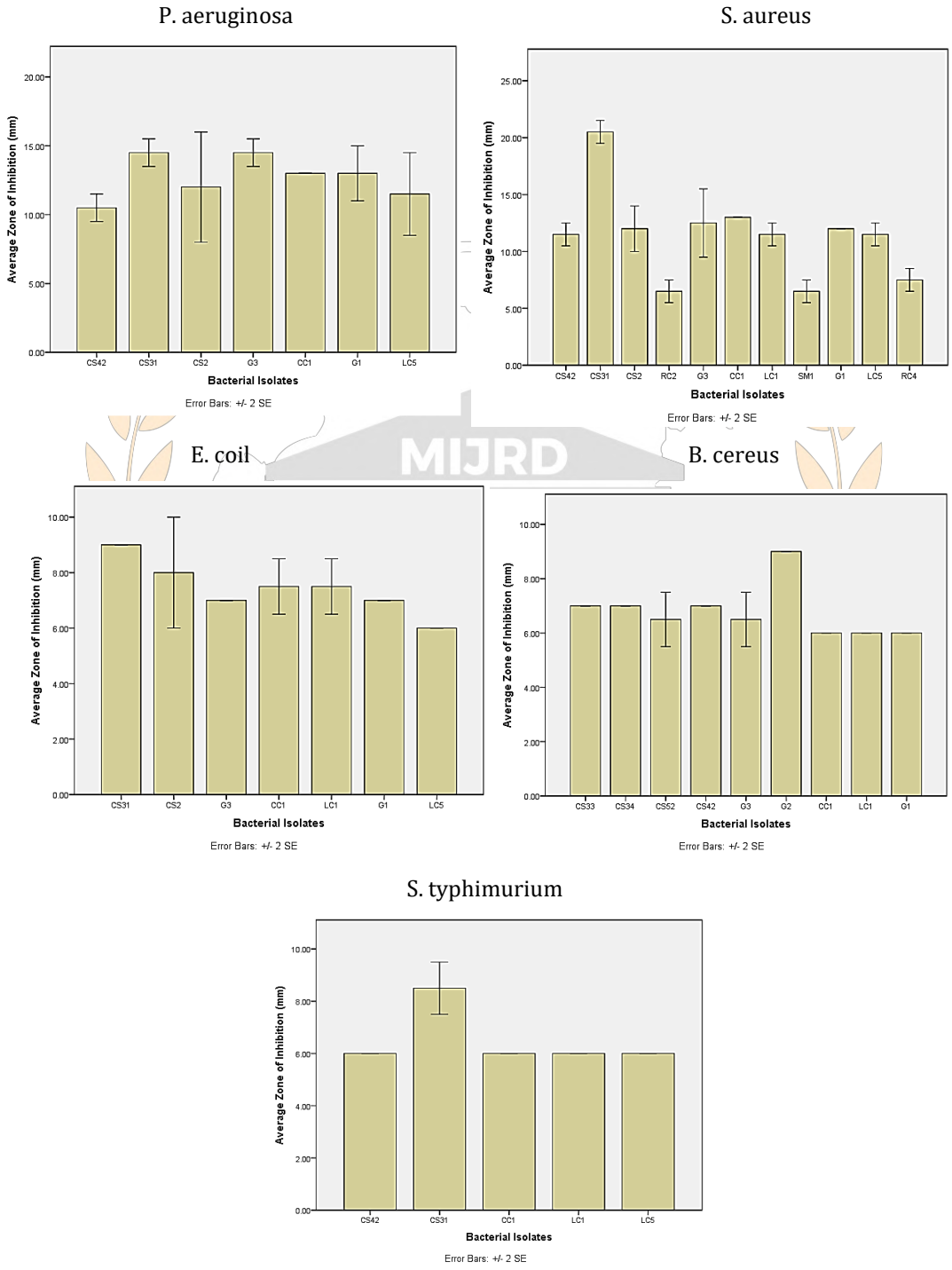


Figure 3. Average zone of inhibition of bacterial isolates against selected pathogenic microorganisms.

Statistical analysis has shown that the activity of the isolates capable of inhibiting P. aeruginosa has no significant difference from each other (P = 0.200). For the activity against S. aureus, it has been shown that the activity of isolate CS31 is significantly high compared to other isolates. The activity of isolates CS31, CS2, CC1, and LC1 is not significantly different from each other. On the other hand, the activity of isolate G2 against B. cereus is significantly different compared to other isolates. And lastly, for the activity against S. typhimurium, isolate CS31 showed significant activity compared to other isolates.

In this study, Bacillus species displayed a promising antagonistic activity against pathogenic organisms. Studies have shown that Bacillus species are well known for their wide range production of pathogen-inhibiting compounds, such as nonribosomal peptides synthetases (NRPs) that exhibit a broad range of activity (Lopes et al., 2018; Miljaković et al., 2020; Hamdache et al., 2013).

Based on the results of this study, it has shown that bacterial isolates isolated from a cave environment can be a good source of bioactive compounds that can have applications in agriculture, pharmaceutical, and other industries.

IV. CONCLUSION AND RECOMMENDATION

Seventeen bacterial isolates were identified and characterized in terms of enzymatic activity, phosphate solubilizing activity, and formaldehyde resistance. Based on 16S rDNA sequencing, the most prevalent isolates obtained were members of the Bacillus genera. Results have shown that 11 out of 17 isolates exhibited amylase activity, and 12 had cellulase activity, showing its potential for industrial application. Eight isolates were positive for phosphate solubilization, which can be used for plant growth promotion and heavy metal immobilization. It was also shown that 13 of 17 isolates could grow under the presence of formaldehyde showing its potential application for the bioremediation of formaldehyde. Some of the bacterial isolates also showed great potential for antimicrobial activity against pathogenic bacteria, specifically the activity of CS31 against P. aeruginosa, E. coli, S. aureus, and S. typhimurium. The results have demonstrated the importance of cave bacteria for the production of valuable bioactive compounds that are commercially important. It is recommended that the quantification of produced secondary metabolites should be performed.

ACKNOWLEDGEMENT

This study was funded by Partido State University through its internal grant for Research and Development. It was also supported by the Department of Science and Technology – Philippine Council for Industry, Energy and Emerging Technology Research and Development (DOST-PCIEERD) through the project entitled "Establishment of ParSU-MTL (Microbiology Testing Laboratory).

REFERENCES

[1] T.R.R. Bontognali, I.M. D'Angeli, N, Tisato, C. Vasconcelos, S.M. Bernasconi, E.R.G. Gonzales and De J. Waele, "Mushroom speleothems: stromatolites that formed in the absence of phototrops" Frontiers of Earth Science, v. 4, no. 49, p.1–8. 2016, https://doi.org/10.3389/feart.2016.00049.

[2] A.S. Engel, "Microbial diversity of cave ecosystems". In: Barton, L.L., Mandl, M. and Loy, A. (eds) Geomicrobiology: Molecular and Environmental Perspective. Springer, the Netherlands, pp. 219–238, 2010.

[3] Y. Gulecal-Pektas, "Bacterial diversity and composition in oylat cave (Turkey) with combined sanger/pyrosequencing approach" Pol. J. Microbiol, 65:69–75, 2016.

[4] A. Hamdache, R. Azarken, A. Lamarti, "Comparative genome analysis of Bacillus spp. and its relationship with bioactive nonribosomal peptide production" Phytochem Rev;12:685–716, 2013.

[5] S. Harirchi, T. Sar, M. Ramezani, H. Aliyu, Z. Etemadifar, S. A. Nojoumi, F. Yazdian, M. K. Awasthi, M. J. Taherzadeh. "Bacillales: From Taxonomy to Biotechnological and Industrial Perspectives" Microorganisms, 10(12):2355, 2022, https://doi.org/10.3390/microorganisms10122355,

[6] K. Tomczyk-Żak &U. Zielenkiewicz, "Microbial Diversity in Caves" Geomicrobiology Journal, 33:1, 20-38, 2020, DOI: 10.1080/01490451.2014.1003341,

[7] J. J. Kim, Y. K. Kwon, J. H. Kim, S. J. Heo, Y. Lee, S. J. Lee, W. B. Shim, W. K. Jung, J. H. Hyun, K. K. Kwon, D. H. Kang, & C. Oh, "Effective microwell plate-based screening method for microbes producing cellulase and xylanase and its application" Journal of Microbiology and Biotechnology, 24(11), 2014, https://doi.org/10.4014/jmb.1405.05052,

[8] R. Lopes, S. Tsui, P. J. R. O. Gonçalves, M. V. Queiroz, "A Look Into A Multifunctional Toolbox: Endophytic Bacillus Species Provide Broad And Underexploited Benefits For Plants" World Journal Of Microbiology And Biotechnology, V. 34, N. 94, P. 4-10, 2018.

[9] D. Miljaković, J. Marinković, & T. S. Balesevic, "The Significance of Bacillus spp. in Disease Suppression and Growth Promotion of Field and Vegetable Crops" Microorganisms. 8. 1037. 10.3390/microorganisms8071037, 2020.

[10] A. Palmer, "Origin and morphology of limestone caves" Geol Soc Am Bull 103: 1-21. Geological Society of America Bulletin - GEOL SOC AMER BULL. 103. 1-21. 10.1130/0016-7606(1991)103<0001:OAMOLC>2.3.CO;2, 1991.

[11] R. Staden, "The Staden Sequence Analysis Package" In Applied Biochemistry and Biotechnology - Part B Molecular Biotechnology, 5(3), 1996, https://doi.org/10.1007/BF02900361 NCB1, 2013

[12] N. Tisato, S. Torriani, S. Monteux, et al., "Microbial mediation of complex subterranean mineral structures" Sci Rep 5, 15525, 2015, https://doi.org/10.1038/srep15525