DOI: http://dx.doi.org/10.15578/iaj.18.2.2023.105-114 *Indonesian Aquaculture Journal, 18 (2), 2023, 105-114*

Available online at: http://ejournal-balitbang.kkp.go.id/index.php/iaj

CHARACTERIZATION OF SPORE-FORMING BACTERIA ISOLATED FROM TILAPIA (*Oreochromis niloticus*) AND THEIR POTENTIAL FOR A PROBIOTIC CANDIDATE

Mira Mawardi[*,], Agustin Indrawati[*)#], Angela Mariana Lusiastuti[***], and I Wayan Teguh Wibawan[*)]**

[*)]Division of Medical Microbiology, School of Veterinary Medicine and Biomedical Sciences, IPB University, Bogor 16680, Jawa Barat, Indonesia

[**)]Government of Indonesia Ministry of Marine Affairs and Fisheries, Main Center for Freshwater Aquaculture, Ministry of Marine Affairs and Fisheries, Sukabumi, Jawa Barat 43114, Indonesia

[***)]Research Center for Veterinary Sciences. National Research and Innovation Agency, KST BRIN Soekarno Cibinong Bogor 16911. Jawa Barat. Indonesia

(Received: May 5, 2023 Final revision: July 28, 2023; Accepted: July 28, 2023)

ABSTRACT

Gram-positive spore bacteria are widely used as probiotics in general sectors. However, there are still limited bacterial isolates as probiotic candidates available from indigenous isolates, especially in aquaculture. This study aimed to obtain potential spore-forming isolates as probiotic candidate for tilapia. Tilapia fish samples were collected from Sukabumi, Ciamis, Serang, and Papua. Bacterial isolates were isolated from the digestive tract of tilapia. Bacteria were identified based on their morphological, molecular characteristics, complete genome composition, and cell surface identification based on hydrophobic properties. In this study, six bacteria were isolated and identified by molecular characteristics using 16S rRNA sequences. Based on the phylogenetic analysis, the 9 PP isolate was Priestia megaterium basonym: *Bacillus megaterium*, CMS 16N isolate was Brevibacillus halotolerans, PPN 10 isolate was *Bacillus sp*., 3.1 SKBM isolate was *Bacillus mycoides*, CMS 22 N and SRG32 isolate were *Bacillus subtilis*. Six bacteria had different phenotypicals, ATGC sequence compositions, and a higher proportion of total G~C sequence composition above 50%. The coherent cell surface hydrophobicity test was positive on the SAT, SA, AA, and compact growth patterns in soft-agar media for 9 PP, CMS 22 N, and SRG32 isolates. From our study, the indigenous spore-forming bacteria isolated from tilapia stomachs are enzymatic bacteria, which have a strong attachment to host tissue and high potential as a probiotic candidate for fish. Various hydrophobicity test results from each isolate indicate that the protein composition in the cell surface is different.

KEYWORDS: ***Bacillus megaterium, Brevibacillus halotolerans, Bacillus mycoides, Bacillus subtilis*, probiotic; tilapia fish**

INTRODUCTION

Probiotics are very popular applied in aquaculture field and most of them are bacteria. Approximately, 27% of Gram-positive bacteria are not pathogenic (Fyzul & Austin, 2015; Alayande *et al*., 2020) and have potential as bio-preservatives, health modulators, antimicrobials, and growth promoters (Turnip *et al*., 2018; Martínez *et al*., 2019; O'Connor *et al*., 2020; Rendueles *et al*., 2022). *Bacillus* is a group of bacteria commonly used as probiotics (Soltani *et al*., 2019), which can produce enzymes and antibiotics, such as *Bacillus megaterium*, *Brevibacillus* sp, *Bacillus mycoides*, and *Bacillus subtilis*/ *Bacillus amyloliquefaciens*. These bacteria have several advantages, so they are widely used in various industries through the biotechnological application.

Bacillus megaterium is one of the most commonly used bacteria in industries due to producing several enzymes (exoenzymes) and metabolic genes for bioremediation (Huang *et al*., 2022; Faraji *et al*., 2022), besides capable of utilizing carbon sources as biocontrol (Goswami *et al*., 2018). Deng *et al.* (2021) stated that the application of *Bacillus megaterium* 1259 in bull could promote growth performances, protein digestibility, rumen fermentation, and nitrogen utilization. *Bacillus megaterium* has also been

[#] Correspondence: Division of Medical Microbiology, School of Veterinary Medicine and Biomedical Science IPB University, Bogor 16680, Jawa Barat
E-mail: indraseta@apps.ipb.ac.id

used for genetic manipulation (Grage *et al*., 2017).

Brevibacillus genus of the Paenibacillaceae family and was first proposed by Shida *et al*. (1996) and this genus has ten officially-registered species (www.bacterio.net/brevibacillus.html). The *Brevibacillus* sp. can produce a variety of enzymes that can inhibit the growth of other bacteria and have the potential as an antimicrobial peptide (Song *et al*., 2012; Che *et al*., 2015; Yang & Yousef, 2018; Atipairin *et al*., 2022). *Brevibacillus* sp. has various functionss as an antifungal material (Jiang *et al*., 2015; Joo *et al*., 2015; Cochrane & Vederas, 2016; Dunlap & Johnson, 2019), a biocontrol agent, and a polyethylene-degrading agent (Panda *et al*., 2014).

Bacillus mycoides are bacteria that have many adaptive genes, which can quickly adapt to environmental condition changes (Fiedoruk *et al*., 2021), in addition to possessing various colony morphotypes. The exopolysaccharide produced by *Bacillus mycoides* has the potential as a biological control agent and an antifungal (Guerrero-Barajas *et al*., 2020), and an antitumor (Farag *et al*., 2020). Yu-Hsiang *et al.* (2017) stated that the zoosporicidal biosurfactant produced by *Bacillus mycoides* could inhibit pythium damping-off disease in cucumber plants. Various enzymes can be produced by *Bacillus mycoides*, for biodegradation and bioethanol production (Rath *et al*., 2022).

Other spore-forming bacteria as a probiotic candidate, that has been widely used in various industrial applications, such as medical, food and feed are *Bacillus subtilis*, due to easier genetic manipulation, easier for large-scaled culture application, producing the largest protein secretion, and safer (Zhang *et al*., 2020). This is because *Bacillus subtilis* produces exopolysaccharide which has a compact film-like structure with high ability as an antibacterial agent activity against *Aeromonas hydrophila*, *Pseudomonas aeruginosa,* and *Streptococcus faecalis*, and antiviral agent (Hassan & Ibrahim, 2017; Lee *et al*., 2016; Caulier *et al*., 2019), besides capable of inhibiting the growth and production of toxins from *Bacillus cereus* (Eom & Choi, 2016). The exopolysaccharide produced by *Bacillus subtilis* has the potential as strong antioxidant and anticoagulant, non-toxic material (Sathishkumar *et al*., 2021), antibacterial and antifungal agent (Camacho *et al*., 2022). According to Zhang & Yi (2022), *Bacillus subtilis* produces extracellular material in large quantities to increase phagocytosis and lysozyme activity in macrophages, then strengthening the immune system and inhibiting cancer cell growth, as well as anti-inflammatory agent. Another advantage of *Bacillus subtilis* is a poly-y-glutamic acid producer, which is non-toxic material and can be used as a cryoprotectant for probiotics (Bhat *et al*., 2013).

Various potentials and benefits of *Bacillus* bacteria have been widely used in various fields. In this study, various isolates were collected for indigenous isolate utilization in aquaculture. Our study aimed to isolate and characterize spore-forming Gram-positive bacteria from the digestive tract of tilapia, which have the potential as probiotic candidates for fish.

MATERIALS AND METHODS

Sample Collection

Bacterial isolate samples were collected from several areas and have been characterized as producing digestive enzymes as published by Mawardi *et al*. (2023). Six of the isolates that have been collected are 9 PP, CMS 16 N, 3.1 SKBM, PPN 10, CMS 22 N, and SRG 32 characterized in this present publication.

Morphological analysis

Isolates were inoculated on Nutrien Agar (Oxoid) medium and incubated at 35°C for 48 hours. The morphology of the bacterial colonies was observed for their color, size, shape, texture, and elevation.

Genotypic and Complete Genome Analysis and Characterization

Quadrant-streaked bacterial isolates on Nutrient Agar (Oxoid) media were sent to PT. Genetika Science Indonesia (www.ptgenetika.com), to obtain complete genome identification and analysis by molecular methods. Phylogenetic analysis was carried out based on the sequencing results using 16S rRNA gene primer pairs. The nucleotide sequence sample data were compared to the GeneBank database at the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov). For the complete genome analysis, a cloning method was performed using *Escherichia coli* DH5á bacteria. The calculation of ATCG composition and percentage of G~C composition was carried out using the software https://www.sciencebuddies.org/science-fair-projects/references/genomics-g-c-content-calculator. A detailed testing procedure followed the method by PT. Genetika Science Indonesia.

Cell Surface Properties

The properties of cell surface was identified to determine the aggregation of bacterial cell surface hydrophobicity, following Mattos-Guaraldi *et al*. (1999). This test was composed of a salt aggregation test, spontaneous aggregation test, automatic aggregation test, bacterial aggregation test with n-hexadecane, and bacterial adherence test to polystyrene material. Bacterial suspension in water was pro-

duced at 10^{11} CFU.

Salt aggregation test (SAT). The formation of clumps was observed from bacterial suspensions and different ammonium sulphates were $(NH_4)2SO_4$ (*Merck*) concentrations. The graded concentration of ammonium sulphate was created between 0.3 M – 4.0 M. The test results can be categorized as; <1.0 M strong/positive, 1.0<2.0 M moderate/positive, 2.0<4.0 M weak/negative, and d"4.0 M non-hydrophobic/negative.

Spontaneous bacterial aggregation test/The spontaneous aggregation (SA). Bacteria were cultured in TSB media and incubated for 24 hours at 37^{0}C, before homogenization. The test results containing many or few clumps of bacteria were categorized as positive, while no lumps/fine suspensions were categorized as negative.

Auto-aggregation (AA)/bacterial aggregation test. The 20 µl of bacterial suspension was mixed with the 20 µl of PBS on the object glass. Formation of bacterial aggregation was observed by rotating the object glass for a minute, while no aggregation formed was categorized as negative.

Bacterial aggregation test with n-hexadecane/Microbial adherence to n-hexadecane test (MATH). The bacterial suspension was mixed with n-hexadecane (10:1) and incubated in a water bath at 37^{0}C for 10 minutes. The sample was mixed using a vortex, then, the Optic Density (OD) on OD_{640nm} was measured on bacterial suspension before and after adding the n-hexadecane. The OD_{640nm} value of the solution before mixing with n-hexadecane was the aqueous phase value. The percentage of hydrocarbons in OD_{640nm} was calculated as [OD_{640nm} (original bacterial suspension) - OD_{640nm}) (aqueous phase)/ OD_{640nm} (original bacterial suspension) x 100]. From this calculation, the hydrophobic value ³50% was qualified as strong/positive, >20 - <50% was qualified as moderate/positive, and d" 20% was qualified as not hydrophobic/negative.

Adhesion test on polystyrene materials/Polystyrene adherence. The 100 µl of bacterial suspension was dropped on a Petri dish made of polystyrene and allowed it to dry at room temperature in a vertical position. The cup surface was rinsed using water, fixed using methanol (Merck), and stained with 1% crystal violet solution (Merck). During the procedure, non-adherent microorganisms were washed off. To identify the properties, bacteria were observed under the microscope. Bacteria with strongly and weakly bound had positive adhesion, while non-bound bacteria had negative adhesion.

The growth pattern of bacterial colonies on soft agar

Soft agar media were prepared with Brain Heart Infusion Broth (BHI, Oxoid) and 0.15% agar (Himedia). The bacterial suspension was inoculated in the soft-agar medium. Bacterial suspension and soft agar were then mixed using a medium-speed vortex. This mixture was incubated at 35°C for 24 hours. The pattern of bacterial growth forms was observed (Wibawan & Lammer, 1990).

RESULTS AND DISCUSSION

Morphological analysis

After the incubation period, each bacterial colony from different isolates had different colors, shapes, sizes of colonies, elevations, and textures (Table 1).

Table 1. Morphology of six bacterial colonies isolated from the digestion tract of tilapia collected from several locations

Isolate	Color	Shape	Size (mm)	Elevation	Texture
9 PP	yellowish	Regular	2-3	Convex	Sticky
CMS 16N	Cream	Irregular	3-5	Flat	Non-sticky
3.1 SKBM	Cream	Regular	3-5	Flat	Non-sticky
PPN 10	Cream	Irregular	3-5	Flat	Non-sticky
CMS 22N	White	Regular	2-3	Convex	Sticky
SRG 32	White	Irregular	2-3	Convex	Sticky

The results of gram staining on all isolates were Gram-positive and the results of spore staining showed the presence of spores on the bacterial cells. The length size of the bacterial cells in each isolate is quite varied ranging from 2.15 ìm to 6.19 ìm. The isolates are motile and non-motile, as shown in Table 2.

Genotypic and Complete Genome Analysis and Characterization

Based on biochemical tests using molecular sequencing tests, similar of species were clearly classified. Based on the identification several samples indicated the same species, but the phylogenetic tree

analysis (Figure 1) showed a different status. In detail, the 3.1 SKBM isolate was different from the PPN 10 isolate, and the CMS 22 N was different from the SRG 32 isolate. Colony and cell morphology were also indicated several differences. The 3.1 SKBM and PPN 10 isolates were identified as *Bacillus mycoides*, but both had no differences in accession number and identity percentage. The PPN 10 isolate had an identity percentage of 95.32% which was different from *Bacillus mycoides* Accession Number MW959783.1, while the 3.1 SKBM isolate had an identity percentage of 99.86% with Accession Number JN9998441. Likewise, the CMS 22 N and SRG 32 isolates showed the same identification at the species level. However, observations of colony, cell morphology, and biochemical tests showed both were different.

Table 2. Cell morphology spore-forming bacteria from six isolates

Isolate	Gram-staining	Spore-staining	Cell size (µm)	Form	Motile
9 PP	Positive	Spore	4.14 – 6.19	Bacilliform	Non-motile
CMS 16 N	Positive	Spore	2.69 – 4.37	Bacilliform	Non-motile
3.1 SKBM	Positive	Spore	3.92 – 4.34	Bacilliform	Non-motile
PPN 10	Positive	Spore	2.91 – 3.12	Bacilliform	Non-motile
CMS 22 N	Positive	Spore	2.44 – 3.10	Bacilliform	Motile
SRG 32	Positive	Spore	2.15 – 2.75	Bacilliform	Motile

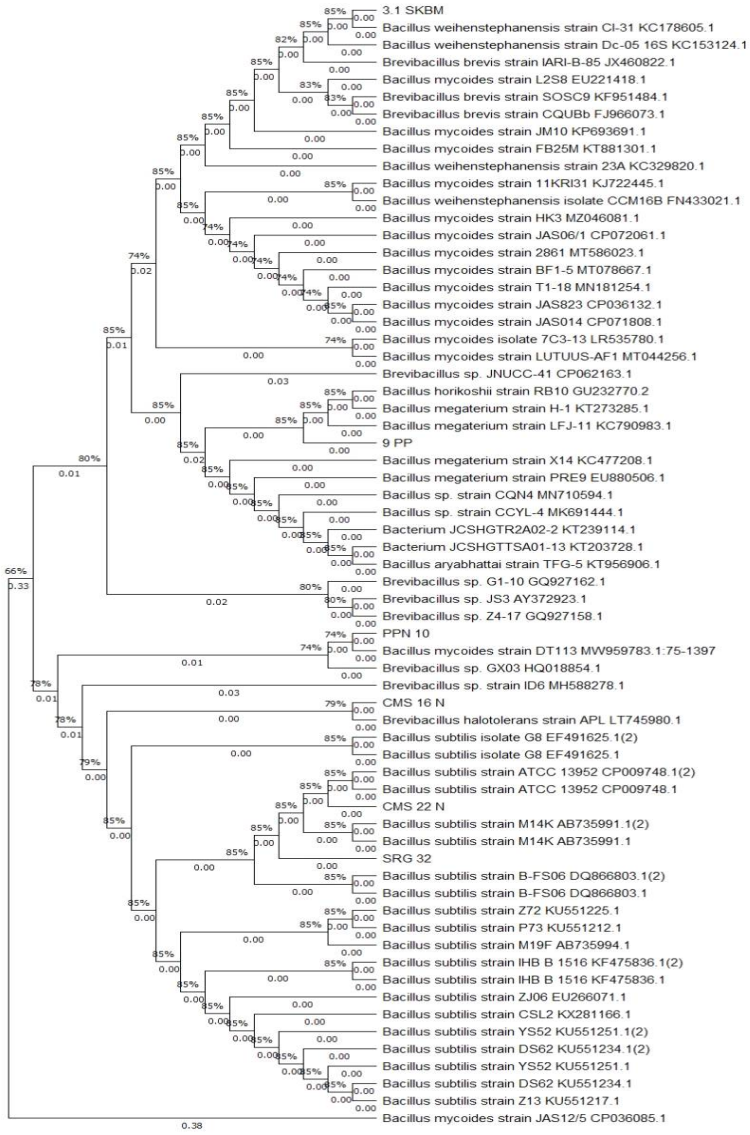

Figure 1. The phylogenetic tree of six isolates presents the relationship and kinship based on the NCBI database using 16S rRNA gene sequences.

The complete sequence analysis for each isolate showed different amounts of Adenine (A), Thymine (T), Guanine (G), and Cytosine (C) bases (Table 3). In the 3.1 SKBM and PPN 10 isolates, the number of ATGC sequences and % G~C were different. The CMS 22 N and SRG 32 isolates also showed a different number of ATGC bases, but the % G~C remained the same. The differences were also indicated by colony morphology, and cell morphology.

Cell Surface Properties

The hydrophobicity test for the SAT, SA, AA, MATH, and adhesion to the polystyrene material on six isolates produced various responses (Table 4). The hydrophobicity test for ammonium sulphate on the six isolates yielded five positive isolates. The 9PP, CMS22N, and SRG32 isolates had the ability to spontaneously be cultured in TSB media (SA test). The 9PP isolate demonstrated the best adhesion capability in n-hexadecane. The 3.1 SKBM isolate had perfect adhesion to polystyrene materials, while the 9 PP, CMS 22 N, and SRG 32 isolates had less adhesion capability.

The growth pattern of bacterial colonies in soft-agar medium

The growth pattern of bacterial colonies in CMS16N and 3.1SKBM isolates display non-compact/undiffuse conditions in soft-agar media (Figure 2), which indicated that the bacteria did not have aggregation properties. Colonies of PPN10 isolates showed obviously compact growth pattern. A similar was also performed by other isolates including 9 PP, CMS 22N, and SRG32, but the colony texture growth in soft-agar media was smoother or lighter.

Table 3. Characterization of ATGC, % G~C and identification of bacteria

Isolate	A	T	G	C	% G~C	Genotypic identification	
9 PP	314	389	347	464	53.6	*Priestia megaterium*	100% OM910720.1
CMS 16 N	301	376	359	476	55.2	*Brevibacillus halotolerans*	99.07% LT745980.1
3.1 SKBM	317	387	341	469	53.5	*Bacillus mycoides*	99.86% JN9998441
PPN 10	300	372	357	471	55.2	*Bacillus mycoides*	95.32% MW959783.1
CMS 22 N	301	376	361	476	55.3	*Bacillus subtilis*	99.60% CP009748.1
SRG 32	377	300	478	360	55.3	*Bacillus subtilis*	99.60% CP009748.1

Table 4. Hydrophobicity test on cell surface of six spore-forming bacteria isolates

Isolate	SAT	SA	AA	MATH	Polystyrene adherence
9 PP	0.3M/Positive	Positive	Positive	42.23%/Positive	Positive
CMS 16 N	2.8M/Negative	Negative	Negative	39.10%/Positive	Negative
3.1 SKBM	0.3M/ Positive	Negative	Positive	16.86%Negative	Positive
PPN 10	0.3M/ Positive	Negative	Negative	11.86%/Negative	Negative
CMS 22 N	0.6M/ Positive	Positive	Positive	15.56%/Negative	Positive
SRG 32	0.6M/ Positive	Positive	Positive	37.30%/Positive	Positive

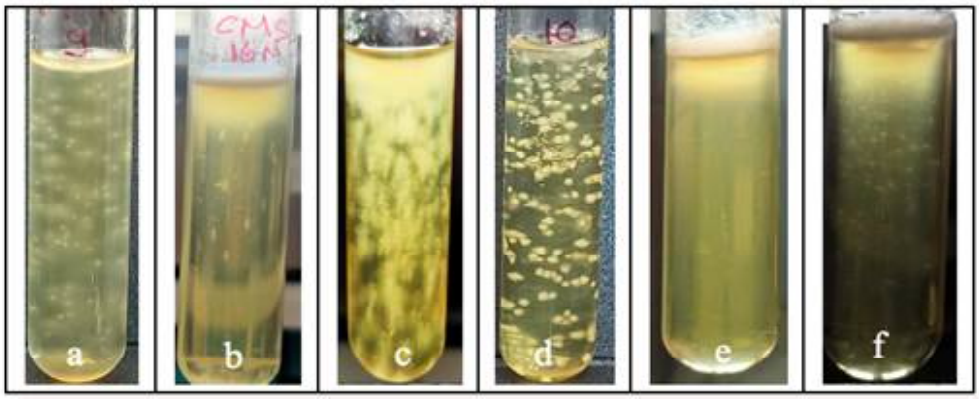

Figure 2. Pattern of bacterial growth in soft agar media culture. (a) 9 PP, (b) CMS 16N, (c) 3.1 SKBM, (d) PPN 10, (e) CMS 22N, and (f) SRG 32 isolates.

The life of living things and the environment will never exist without the role of microorganisms including bacteria. Bacteria have important roles in the fish digestive tract to produce extracellular enzymes to degrade food in digest tract. According to Hussain *et al*. (2017), the cellulolytic bacteria of *Bacillus amyloliquefaciens* subsp. *plantarum*, *Bacillus megaterium*, *Bacillus subtilis subtilis* subsp. *subtilis*, and *Anoxybacillus flavithermus*, can be isolated from soil and poultry. Cellulolytic bacteria generally produce the exoglucanase, endoglucanase, and â-glucosidase (Karthika *et al*., 2020).

The six isolates were selected based on several criteria including they are categorized vegetative cell bacteria which have long lifetime, more stable and resistant to temperature and high pH, and are resistant to feed pelletization processes (Purwandari & Chen, 2013). The spore are resistant to heat at 80°C for 10 minutes (Peet *et al*., 2015).

Six bacterial samples belong to the genus *Bacillus*, with having spores. Furthermore the molecular genome method indicated that the PPN 10 isolate had an identification percentage value of 95.32% to *Bacillus mycoides* MW959783.1 as enlisted on the NCBI GeneBank. According to Stackebrandt and Goebel (1994), the species identifications are considered similar when the identity value percentage is above 97.5%, similar for genus level if the identity percentage value is above 95%. Therefore, the PPN 10 isolate was genotypically identified as *Bacillus* sp., the results PPN 10 and 3.1 SKBM isolates were categorized bacterial isolates with a different species. Other indicators shown the differences of the two isolates were the differences in the colony and cell morphology. The 3.1 SKBM and PPN 10 isolates are not only phenotypically different but also have different numbers of ATGC genome sequences (Table 3). The PPN 10 and 3.1 SKBM isolates also had differences in cell surface hydrophobicity, following the automatic aggregation test and hydrophobicity test for polystyrene materials. The 3.1 SKBM isolate had strong hydrophobic properties in the automatic aggregation test by adhering strongly to polystyrene materials but was not correlated with the aggregation results in the soft-agar method and hydrophobic test on n-hexadecane.

The complete sequence composition of six isolates showed the different amount of ATGC. The results demonstrated the highest G~C sequencer composition of six isolates above 50% where, the CMS 22N and SRG 32 isolates had the highest value of 55.3%. According to Garg & Sharma (2020), the C phosphodiester G Island (CpGI) sequence is a DNA sequence region in DNA that has a minimum concentration of G~C of 50%. The CpG DNA is well-known as adjuvant immunity (Xue *et al*., 2019). The CpG dinucleotides are flanked by certain bases, that can function as non-self-pattern recognition motifs for innate immune system receptors to detect the infecting pathogens (Seya & Matsumoto, 2019). According to Bach *et al*. (2022), the *Bacillus* genus has an average mole G~C of 32 - 69%.

The CMS16N isolate was genotypically identified as *Brevibacillus halotolerans* LT745980.1 at 99.07 %. According to Song *et al*. (2017), *Brevibacillus halotolerans* strains LAM0313T and LAM0313 are Gram-positive, spore-forming, rod-shaped, and peritrichous flagella bacteria with G~C composition of 45.0% and 46.0% and can grow on media added with NaCl 12%. Choi *et al*. (2019) described that *Brevibacillus antibioticus* sp. nov has a broad potential for antibacterial activity against several pathogenic bacteria with G~C composition of 47.0 %. Moreover, Kong *et al.* (2014) reported that *Lysinibacillus halotolerans* strain LAM612(T) has G~C content of 36.4% and can survive in media with NaCl 10%. The *Brevibacillus* genus has G~C composition of 40.2 – 57.4% (Logan and De Vos, 2015). In this study, the CMS 16N isolate had G~C composition of 55.2% and produced amylolytic and proteolytic enzymes.

Priestia megaterium, basonym: *Bacillus megaterium* has plasmids that can produce bacteriocins with G~C composition of 52 % (Harirchi *et al*., 2022). Based on Shwed *et al.* (2021), % G~C of *Priestia megaterium* strain ATCC 14581T ranges from 33.6 % - 38.4 %, the CDC strain 2008724129 ranges is from 33.7 % - 39.0 % and strain 2008724142 from 33.0 % - 38.1 %. *Bacillus megaterium* is non-pathogenic bacteria and has been commercially available for biotechnological application, such as vitamin B12, penicillin acylase and amylase with G~C composition of 38.2%. In this study present, *Priestia megaterium* identified from the 9 PP isolate had a higher G~C composition of 53.6%. This isolate could utilize lipid and protein indicated by its higher ability to degrade protein than other isolates. The 9 PP isolate had strong adherent properties, and showe a hydrophobic cell surface.

Hydrophobicity of bacterial cell surface is a biological phenomenon that varies greatly among species. This biological phenomenon illustrates the interaction between bacteria and their host tissue, bacteria in solution, or bacteria and phagocytes. In this study present, the cell surface hydrophobicity of each bacterial isolate showed different results in each aggregation test; SAT, SA, AA, MATH, and adherence to polystyrene materials. The test method also produced different analysis variations on the cell surface hydrophobic test in *Staphylococcus epidermidis* and

found indications that adherence to polystyrene materials was affected by the involvement of the bacterial cell surface protein components (Krepsky *et al*., 2003). Bacterial clumping in n-hexadecane is a rapid method for determining cell surface hydrophobicity, which can be mediated by surface proteins or glycolipid complexes in surface protein structures. The 9 PPN isolate was observed to have higher hydrophobicity than other isolates in n-hexadecane material at 42.23%. The results of the hydrophobicity test on CMS 22N and SRG 32 isolates were found similar to Ritter *et al*. (2018), that *Bacillus subtilis* has adhesive properties on hydrocarbon materials at 2.2-56.4% and 4.4-52.9% for auto-aggregation. The CMS 22 N and SRG 32 isolates had hydrophobic properties in the n-hexadecane material in a negative (15.56%) and positive (37.30%) conditions as found in this present study. The CMS 22 N and SRG 32 have positive correlation on SAT, SA, AA, and polystyrene materials. The aggregation properties of bacteria were also demonsrated by the results of growth tests in soft-agar media (Figure 2). According to Wibawan and Lämmler (1991), the growth pattern of compact bacterial colonies in the soft-agar medium is determined by the cell surface protein components as aggregation properties. The relevance of cell surface hydrophobicity test and the pattern of colony growth in soft agar medium are influenced by the component structure of the cell surface. The 9 PP, PPN 10, CMS 22 N, and SRG 32 isolates performed compact growth patterns in a soft medium, indicating that these isolates have aggregation and coherent properties with the hydrophobicity test with using SAT method.

CONCLUSIONS

Based on the characteristics of the hydrophobic cell surface and complete genome discovered in this present study, six bacteria are qualified as probiotic candidates for fish by having SAT, SA, AA, and polystyrene materials specific.

ACKNOWLEDGMENTS

This study was funded by grants from the Pascasarjana Doctoral Program Research of the Indonesian Ministry of Education, Culture, Research, and Technology, contract number 18830/IT3.D10/PT.01.02/M/T/2023. The authors would like to honor Mr. Fernando Jongguran Simanjuntak, the Director of the Main Center for Freshwater Aquaculture Laboratory, for granting permission to use the facilities. The authors gratefully thank the School of Veterinary Medicine and Biomedical Sciences for allowing them to conduct laboratory works and staffs and the laboratory analysts for their cooperation.

REFERENCES

Alayande, K. A., Aiyegoro, O. A., & Ateba, C. N. (2020). Probiotics in animal husbandry: applicability and associated risk factors. *Sustainability Journal*. 12(3),1087. https://doi.org/10.3390/su12031087

Atipairin, A., Songnaka, N., Krobthong, S., Yingchutrakul, Y., Chinnawong, T., & Wanganuttara, T. (2022). Identification and characterization of a potential antimicrobial peptide isolated from soil *Brevibacillus* sp. WUL10 and Its activity against MRSA pathogens. *Trop Med Infect Dis Jur* 7;7(6),93. https://doi.org/10.3390/tropicalmed7060093.

Bach, E., Rangel, C. P., Ribeiro, I. D. A., & Passaglia, L .M. P. (2022). Pangenome analyses of *Bacillus pumilus*, *Bacillus safensis,* and *Priestia megaterium* exploring the plant-associated features of bacilli strains isolated from canola. *Molecular Genetics and Genomics*,297:1063–1079. doi: 10.1007/s00438-022-01907-0

Bhat, A. R., Irorere, V. U., Bartlett, T., Hill, D., Kedia, G., Morris, M. R., Charalampopoulos D., & Radecka, I. (2013). *Bacillus subtilis* natto: a non-toxic source of poly-ã-glutamic acid that could be used as a cryoprotectant for probiotic bacteria. *AMB Express*.3, 36. doi: 10.1186/2191-0855-3-36

Camacho, M. I., García, J. M., Rogen, D., Ferrer, A., Wieme, A. D., Vandamme, P., Rodríguez, S., … & Meriño, T-L,. (2022). Isolation and identification of a *Bacillus* sp. from freshwater sediment displaying potent activity against bacteria and phytopathogen fungi. *Current Microbiology*,79(12), 398. doi: 10.1007/s00284-022-03090-2.

Caulier, S., Nannan, C., Gillis, A., Licciardi. F., Bragard, C., & Mahillon. (2019). Overview of the antimicrobial compounds produced by members of the *bacillus subtilis* group. *Frontiers in Microbiolgy*.10-2019. https://doi.org/10.3389/fmicb.2019.00302

Che, J., Liu, B., Ruan, C., Tang, J., & Huang, D. (2015). Biocontrol of *Lasiodiplodia theobromae*, which causes black spot disease of harvested wax apple fruit, using a strain of *Brevibacillus brevis* FJAT-0809-GLX. *Crop Prot* 67:178–183. DOI:10.1016/j.cropro.2014.10.012

Choi, A., Nam, Y. H., Baek, K., & Chung, E. J. (2019). *Brevibacillus antibioticus* sp. nov., with a broad range of antibacterial activity, isolated from soil in the Nakdong River. J *Microbiol*. 57(11), 991-996. doi: 10.1007/s12275-019-9325-y

Cochrone, S.A., & Vederas, J.C. (2016). Lipopeptides from Bacillus and *Paenibacillus* spp.: a gold mine

of antibiotic candidates, *Medicinal Research Reviews*, 36(1), 4-31. doi: 10.1002/med.21321.

Deng, B., Chen, Y., Gong, X., Dai, Y., Zhan, K., Lin, M., Wang, L., & Zhao, G. (2021). Effects of *Bacillus megatherium* 1259 on growth performance, nutrient digestibility, rumen fermentation, and blood biochemical parameters in holstein bull calves. *Animals Journal*. 11(8), 2379. https://doi.org/10.3390/ani11082379

Eom, J. S., & Choi, H. S. (2016). Inhibition of *Bacillus cereus* growth and toxin production by *Bacillus amyloliquefaciens* RD7-7 in Fermented Soybean Products. *J. Microbiol. Biotechnol.* 26(1), 44–55. doi: 10.4014/jmb.1509.09090.

Farag, M. M. S., Moghannem, S. A. M., Shehabeldine, A. M., & Azab, M. S. (2020). Antitumor effect of exopolysaccharide produced by *Bacillus mycoides*. *Microb Pathog*,140,103947. doi: 10.1016/j.micpath.2019.103947.

Faraji, F., Mahandra, H., & Ghahreman, A. (2022). Evaluation of different amino acids on growth and cyanide production by *bacillus megaterium* for gold recovery. *Sustainability Journal, 14*(15), 9639. https://doi.org/10.3390/su14159639

Fiedoruk, K., Drewnowska, J. M., Mahillon, J., Zambrzycka, M., & Swiecicka, I. (2021). Pan-Genome Portrait of *Bacillus mycoides* provides insights into the species ecology and evolution. *Microbiol Spectr* 3, 9(1): e00311-21. doi: 10.1128/Spectrum.00311-21

Fyzul, A.N., & Austin, B. (2015). Probiotics, immunostimulants, plant products and oral vaccines, and their role as feed supplements in the control of bacterial fish disease. *Journal of Fish Disease*. 38(11), 937-955. doi: 10.1111/jfd.12313.

Goswami, G., Panda, D., Samanta, R., Boro, R. C., Modi, M. H., Bujarbaruah, K. M., & Barooah, M. (2018). *Bacillus megaterium* adapts to acid stress condition through a network of genes: Insight from a genomewide transcriptome analysis. *Scientific Reports*, 8, 16105. doi: 10.1038/s41598-018-34221-0

Grage, K., McDermott, P., & Rehm, B.H.A. (2017). Engineering *Bacillus megaterium* for production of functional intracellular materials. *Microbial Cell Factories*,16, 211. doi:10.1186/s12934-017-0823-5

Guerrero-Barajas, C., Constantino-Salinas, E.A., Amora-Lazcano, E., Tlalapango-Angeles, D., Mendoza-Figueroa, J., Cruz-Maya, J.A., & Jan-Roblero, J. (2020). *Bacillus mycoides* A1 and *Bacillus tequilensis* A3 inhibit the growth of a member of the phytopathogen *Colletotrichum gloeosporioides* species complex in avocado. *J Sci Food Agric* 100(10), 4049-4056. doi: 10.1002/jsfa.10450.

Harirchi, S., Sar, T., Ramezani, M., Aliyu, H., Etemadifar, Z., Nojoumi SA, Yazdian, F., … & Taherzadeh, M. J. (2022). *Bacillales*: from taxonomy to biotechnological and industrial perspectives. *Microorganisms Journal*, 10(12), 2355. doi.org/10.3390/microorganisms10122355

Hassan, S. W. M & Ibrahim, H. A. H. (2017). Production, Characterization and Valuable Applications of Exopolysaccharides from Marine *Bacillus subtilis* SH1. *Pol J Microbiol* 4, 66(4), 449-461. doi: 10.5604/01.3001.0010.7001.

Huang, Y., Chen, W-Y., Li, J., Ghorab, M. A., Alansary, N., El-Hefny, D. E., El-Sayyad, G. S., … & Chen, S. (2022). Novel mechanism and degradation kinetics of allethrin using *Bacillus megaterium* strain HLJ7 in contaminated soil/water environments. *Environmental Research*, 214(3), 113940.doi: 10.1016/j.envres.2022.113940.

Hussain, A. A., Abdel-Salam, M. S., Abo-Ghalia, H. H., Hegazy, W. K., & Hafez, S.S. (2017). Optimization and molecular identification of novel cellulose degrading bacteria isolated from Egyptian environment. *Journal of Genetic Engineering and Biotechnology*,15(1), 77–85. doi: 10.1016/j.jgeb.2017.02.007.

Jiang, H., Wang, X., Xiao, C., Wang, W., Zhao, X., Sui, J., Sa, R., Guo, T. L., & Liu, X. (2015). Antifungal activity of *Brevibacillus laterosporus* JX-5 and characterization of its antifungal components. *World J Microbiol Biotechnol* 31(10), 1605–1618. doi: 10.1007/s11274-015-1912-4.

Johnson, S. T. & Dunlap, C. A (2019). Phylogenomic analysis of the *Brevibacillus brevis* clade: a proposal for three new Brevibacillus species, *Brevibacillus fortis* sp. nov., *Brevibacillus porteri* sp. nov. and *Brevibacillus schisleri* sp. nov. *Antonie van Leeuwenhoek*,112(7), 991–999. doi: 10.1007/s10482-019-01232-4.

Joo, H. J., Kim, H. Y., Kim, L. H., Lee, S., Ryu, J. G., & Lee, T. (2015). A *Brevibacillus* sp. antagonistic to mycotoxigenic Fusarium spp. *Biol Control* 87, 64–70. https://doi.org/10.1016/j.biocontrol.2015.04.010

Karthika, A., Seeniavasagan, R., Kasimani, R., Babalola, O.O., & Vasanthy, M. (2020). Cellulolytic bacteria isolation, screening and optimization of enzyme production from vermicompost of paper cup waste. *Wase Management* 116, 58-65. doi: 10.1016/j.wasman.2020.06.036

Kong, D., Wang, Y., Zhao, B., & Li, Y. (2014). *Lysinibacillus halotolerans* sp.Nov., isolated from saline-alkaline soil. I*nternational Journal of Systematic and Evolutionary Microbiology*, 64(8):2593-2598. doi: 10.1099/ijs.0.061465-0.

Krepsky, N., Ferreira, R. B. R., Nunes, A. P. F., Lins, U. G. C., Filho, F. Ce. S., Mattos-Guaraldi, A. L de., Netto-dos., & Santos. (2003). Cell surface hydrophobicity and slime production of *Staphylococcus epidermidis* Brazilian isolate. *Current Microbiology*, 46, 280-286. doi: 10.1007/s00284-002-3868-5.

Lee, J. Y., Shim, J. M., Yao, Z., Liu, X., Lee, K. W., Kim, H. -J., Ham, K. -S., & Kim, J. H. (2016). Antimicrobial activity of *Bacillus amyloliquefaciens* EMD17 isolated from *Cheonggukjang* and potential use as a starter for fermented soy foods. *Food Science and Biotechnology*, 25(2), 525–532. doi: 10.1007/s10068-016-0073-z

Logan, N.A., & De Vos, P. (2015). Bacillus. Bergey's Manual of Systematic of Archaea and Bacteria. 2nd edn, vol. 3. New York: Springer, 304–316. https://link.springer.com/book/10.1007/978-0-387-68233-4

Martínez, B., García, P., & Rodríguez, A. (2019). Swapping the roles of bacteriocins and bacteriophages in food biotechnology. *Curr Opin Biotechnol*, 56,1-6. doi: 10.1016/j.copbio.2018.07.007.

Mattos-Guaraldi, A. L., Formiga, L. C., & Andrade, A. F. (1999). Cell surface hydrophobicity of sucrose fermenting and nonfermenting Corynebacterium diphtheriae strains evaluated by different methods. *Current Microbiology* 38(1), 37-42. doi: 10.1007/pl00006769.

Mawardi, M., Indrawati, A., Wibawan, I. W. T., & Lusiastuti, A.M. (2023). Antimicrobial susceptibility test and antimicrobial resistance gene detection of extracellular enzyme bacteria isolated from tilapia (*Oreochromis niloticus*) for probiotic candidates. *Veterinary World*, 16(2), 264–271. doi.org/10.14202/vetworld.2023.264-271

O'Connor, P. M., Kuniyoshi, T. M., Oliveira, R. P. S., Hill, C., Ross, R. P., & Cotter, P.D. (2020). Antimicrobials for food and feed; a bacteriocin perspective. *Current Opinion in Biotechnology*, 61, 160-167. doi: 10.1016/j.copbio.2019.12.023.

Panda, A. K., Bisht, S. S., DeMondal, S., Kumar, N. S, Gurusubramanian, G. G., & Panigrahi, A.K. (2014). *Brevibacillus* as a biological tool: *a short review. Antonie van Leeuwenhoek*,105(4), 623–639. DOI: 10.1007/s10482-013-0099-7

Peet, K. C., Freedman, A. J., Hernandez, H. H., Britto, V., Boreham, C., & Ajo-Franklin, J.B. (2015). Microbial growth under supercritical CO2. *Appl. Environ. Microbiol,* 81(8), 2881–2892. doi: 10.1128/AEM.03162-14

Purwandari, A. R., & Chen, H. Y. (2013). Effects of probiotic *Bacillus subtilis* on intestinal microbial diversity and immunity of orange-spotted grouper *Epinephelus coioides*. *Journal of Applied Biotechnology*, 1(1). https://doi.org/10.5296/jab.v1i1.3714

Rath, S., Paul, M., Behera, K.H., & Thatoi, H. (2022). Response surface methodology mediated optimization of Lignin peroxidase from *Bacillus mycoides* isolated from Simlipal Biosphere Reserve, Odisha, India. *Journal of Genetic Engineering and Biotechnology*, 20:2.https://doi.org/10.1186/s43141-021-00284-2

Rendueles, M. C., Duarte, A. C., Escobedo, S., Rodríguez, L., Rodríguez, A., García, P., & Rodríguez, A. (2022). Combined use of bacteriocins and bacteriophages as food biopreservatives. A review. *J Food Microbiol*.2, 368, 109611.https://doi.org/10.1016%2Fj.ijfoodmicro.2022.109611

Ritter, A. C., Correa, A. P. F., Veras, F. F., & Brandelli, A. (2018). Characterization of *Bacillus subtilis* Available as Probiotics. *Journal of Microbiology Research*, 8(2): 23-32. doi:10.5923/j.microbiology.20180802.01

Sathishkumar, R., Kannan, R., Jinendiran, S., Sivakumar, N., Selvakumar, G., & Shyamkumar, R. (2021). Production and characterization of exopolysaccharide from the sponge-associated *Bacillus subtilis* MKU SERB2 and its in-vitro biological properties. I*nt J Biol Macromol* 1, 166, 1471-1479. https://doi.org/10.1016/j.ijbiomac.2020.11.026

Seya, T., Takeda, Y., & Matsumoto M. (2019). A Toll-like receptor 3 (TLR3) agonist ARNAX for therapeutic immunotherapy. *Advanced Drug Delivery Reviews*,147, 37-43. https://doi.org/10.1016/j.addr.2019.07.008

Shwed, P.S., Crosthwait, J., Weedmark, K., Hoover, E., & Dussault, F. (2021). Complete genome sequences of *Priestia megaterium* type and clinical strains feature complex plasmid arrays. *Microbiol Resour Announc*, 10(27), e00403-21. https://doi.org/10.1128%2FMRA.00403-21

Shida, O., Takagi, H., Kadowaki, K., & Komagata, K. (1996). Proposal for two new genera, *Brevibacillus* gen. nov. and *Aneurinibacillus* gen. nov. *Int J Syst Bacteriol* 1996, 46, 939-946. 10.1099/00207713-46-4-939

Soltani, M., Ghsh, K., Hoseinifar, S. H., Kumar, V., Lymbery, A. J., Roy, S., & Ringø E. (2019). Genus *bacillus*, promising probiotics in aquaculture: Aquatic animal origin, bio-active components, bioremediation and efficacy in fish and shellfish. *Reviews in Fisheries Science & Aqua culture*, 27(3). https://doi.org/10.1080/23308249.2019.1597010

Song, J., Wang, Y., Song, Y., Zhao, B., Wang, H., Zhou, S., Kong, D., … & Yan, Y. (2017). *Brevibacillus halotolerans* sp. nov., isolated from saline soil of a paddy field. *Int J Syst Evol Microbiol*,67(4), 772–77. doi: 10.1099/ijsem.0.001579.

Song, Z., Liu, Q., Guo, H., Ju, R., Zhao, Y., Li, J., & Liu, X. (2012). Tostadin, a novel antibacterial peptide from an antagonistic microorganism *Brevibacillus brevis* XDH. *Biores Technol* 111, 504–506. https://doi.org/10.1016/j.biortech.2012.02.051

Stackebrandt, E., & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA Reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic and Evolutionary Microbiology* 44, 846–849. https://doi.org/10.1099/00207713-44-4-846

Steiner, E., & Margesin, R. (2020). Production and partial characterization of a crude cold-active cellulase (CMCase) from *Bacillus mycoides* AR20-61 isolated from an Alpine forest site. *Annals of Microbiology,* 70, 67. https://doi.org/10.1186/s13213-020-01607-3

Turnip, E. R., Widanarni., & Meryandini, A. (2018). Selection of lactic acid bacteria as a probiotic and evaluated its performance on gnotobiotic catfish *Clarias* sp. Jurnal Akuakultur Indonesia 17(1): 68–80. DOI: 10.19027/jai.17.1.68-80

Wibawan, I. W. T., & Lammler C. (1990). Properties of Group B Streptococci with Protein Surface Antigens X and R. *Journal of Clinical Microbiology,* 28(12): 2834-2836. https://doi.org/10.1128%2Fjcm.28.12.2834-2836.1990

Wibawan, I. W. T & Lammler C. (1991). Influence of capsular neuraminic on properties of streptococci of serological group B. *Journal of General Microbiology* 137(12), 2721-2725. https://doi.org/10.1099/00221287-137-12-2721

Xue, X., Woldemariam, N. T., Caballero-Solares, A., Umasthan, N., Fast, R. G., Taylor, R. G., Rise, M. L., & Andreassen, R. (2019). Dietary immunostimulant cpg modulates microrna biomarkers associated with immune responses in atlantic salmon (*Salmo salar*). *Cells*, 8(12),1592. https://doi.org/10.3390/cells8121592

Yang, X & Yousef, A. E. (2018). Antimicrobial peptides produced by *Brevibacillus* spp.: structure, classification and bioactivity: a mini review. *World J Microbiol Biotechnol,* 34, 57. https://doi.org/10.1007/s11274-018-2437-4

Yu-Hsiang, P., Yun-Jung, C., Yung-Chuan, L., Jen-Fon, J., Kuang-Ren, C., & Jenn-Wen, H. (2017). Inhibition of cucumber Pythium damping-off pathogen with zoosporicidal biosurfactants produced by Bacillus mycoides. *Journal of Plant Diseases and Protection*,124, 481–491. http://dx.doi.org/10.1007/s41348-017-0110-z

Zhang, L., & Yi, H. (2022). Potential antitumor and anti-inflammatory activities of an extracellular polymeric substance (EPS) from *Bacillus subtilis* isolated from a housefly. Sci Rep 26;12(1):1383. https://doi.org/10.1038/s41598-022-05143-9

Zhang, X., Al-Dossary, A., Hussain, M., Setlow, P., & Li, J. (2020). Applications of *Bacillus subtilis* spores in Biotechnology and Advanced Materials. *Applied and Enviromental Microbiology*, 86(17). https://doi.org/10.1128/AEM.01096-20.

