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Agarolytic *Bacillus* sp. FRAgK1 Screened from *Gracilaria* (Rhodophyta) Thallus as Probiotic Candidate for Abalone

Faisal Zain Abdullah[1], Indah Istiqomah[1]*, Alim Isnansetyo[1], Ngurah Sedana Yasa[2] & Norshida Ismail[3]

[1]Department of Fisheries, Faculty of Agriculture, Universitas Gadjah Mada, Sleman Regency, Special Region of Yogyakarta, Indonesia

[2]Research Center for Fisheries, National Agency for Research and Innovation (BRIN), Bogor, West Java, Indonesia

[3]School of Animal Science, Aquatic Science and Environment, Faculty of Bioresources and Food Industry. Universiti Sultan Zainal Abidin, Terengganu, Malaysia

*Corresponding author, e-mail: indah_ist@ugm.ac.id

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ABSTRACT Agarolytic bacteria produce agarase, which may aid in the growth of cultured tropical abalone fed natural seaweed. Agarolytic bacteria can come from a variety of sources, such as seawater, abalone intestines, and dead seaweed. This study aimed to isolate, screen, describe, and identify agarolytic bacteria found in red macroalgae. Agarolytic bacteria isolated from Gracilaria segregated from the substrate at Drini Beach, Gunungkidul Regency, were qualitatively described using the agarolytic index, antibiotic susceptibility, acid resistance (pH 4), and safety test. We collected seven agarolytic isolates. FRAgK1 isolate had the highest agarolytic index, was sensitive to antibiotics, resistant to low pH conditions, and non-pathogenic to finfish, making it suitable for use as gut probiotics in abalone. The bacterium was short rod-shaped, Gram-positive, non-motile, lacked catalase and indol, and was unable to ferment lactose or sucrose. The 16S rRNA gene sequence of FRAgK1 was most like *Bacillus subtilis*, however only by 99.43%.

Keyword: Abalone; agarolytic; *Bacillus*; probiotic;16S rRNA

INTRODUCTION

Abalone is a fishery and aquaculture commodity with growing global market demand, particularly in East Asia (Hernández-Casas et al., 2023). Indonesia is one of the abalone producing countries, with growing business attention to this commodity (Dwi & Hollanda, 2023). Indonesia's abalone production is now concentrated on the *Haliotis asinina* and *Haliotis squamata* species through cultivation initiatives (Grandiosa, 2020). *Haliotis squamata* has been proven to have high resistance to various stressors (Yasa et al., 2020). The main challenge in the abalone culture industry is the slow growth rate. Numerous studies have been performed to increase the growth of abalone (Nurfajrie et al., 2014; Maulidya et al., 2021). It has been observed that the natural diet of macroalgae resulted in better growth rates than existing artificial feed in tropical abalone (Damayanti *et al*., 2018). Tropical abalone feed in Indonesia is primarily made up of macroalgae found in nature, particularly *Gracilaria* sp. (Rhodophyta) and *Ulva* sp. (Chlorophyta) (Yusup et al., 2020).

Several strategies for improving abalone growth performance have been offered, one of which is the use of probiotics (Amin et al., 2020). Probiotics have been extensively examined in the aquaculture business and found to improve growth rates (Cruz et al. 2012; Moonsamy et al., 2020). The application of probiotics to abalone has currently been tried using a microencapsulation approach (Masoomi-Dezfooli et al., 2021). Probiotics may promote organism growth by stabilizing the gut bacterial flora. Red seaweed (Rhodophyta) is a macroalga that mostly contains agar polysaccharides, which are complex hydrocolloid substances. Agar structure is composed of polysaccharide agarose and agaropectin. Agarose (β-D-galactose dan 3,6-anhydrous-α-L-galactose) is the main agar component generally hard to digest. Enzymatic hydrolysis of agar occurs in the digestive tract to aid in the breakdown process of agar by cultured organisms (Chi *et al*., 2012). Agarase is the enzyme responsible for agar hydrolysis, and bacteria that use agar as a carbon source are known as agarolytic bacteria (Kang et al., 2013).

Agarolytic bacteria have been obtained from various sources such as seawaters and bottom sediments (Kim *et al*., 2011; Anggraeni & Ansorge-Schumacher, 2021) abalone digestive tract (Meinita *et al*., 2008), and seaweed (Kawaroe *et al*., 2017; Kandasamy *et al*., 2020). Genera of agarolytic bacteria that have been isolated and produce agarase include Alteromonas, Pseudomonas, Vibrio, Cytophaga, Agarivorans, Thalassomonas, Pseudoalteromonas, Bacillus, dan Acinetobacter (Fu & Kim, 2010). further investigated the use of The agarolytic probiotic *Vibrio natriegens* has been demonstrated to dramatically boost the growth rate of abalone (Faturrahman *et al*., 2015). These findings suggest that the use of probiotics and the search for agarolytic bacteria to aid abalone cultivation in Indonesia holds great promise. the purpose of, which observed an increase in growth rate. The purposes of the present study were to isolate, characterize, and identify agarolytic bacteria from red seaweed which may be useful in probiotic searches.

MATERIALS AND METHODS

Collection and isolation of agarolytic bacteria

The research was performed in the Laboratory of Fish Health and Environment, Gadjah Mada University, Indonesia. A couple of pieces of red seaweed (Rhodophyta) washed ashore on Drini Beach, Gunungkidul Regency, Province D.I. Yogyakarta, Indonesia, were collected along with seawater and then taken safely in a cool box to the laboratory. The sample was crushed septically and then enriched in Zobell Marine Broth medium for 48 h at room temperature using the orbital shaker. Isolation was done by inoculating a diluted sample on Zobell Marine Agar and incubating it at 29 °C for 24 h. Colonies of different morphology were purified by the streak plate method. Purified isolates were streaked fortnightly for daily testing and stored in

20% glycerol Marine Broth (v/v).

Qualitative detection of agarase activity

Agarolytic screening was conducted on Zobell marine agar with Lugol's iodine drop based on clear zones development surrounding the bacterial colony (Zilda *et al*., 2021). All isolates were single pointily inoculated to Marine Agar with only 1.5% agar for a carbon source. Cultures were grown for 72 h to ensure the agar hydrolysis process. The medium then was flooded with 2 ml of Lugol's iodine. Colony diameter and clear zone from iodin were measured.

$$Agarolytic \quad Index = \frac{(Clear \quad zones \quad (mm) - COlony \quad diameter \quad (mm)}{(Colony \quad diameter \quad (mm))}$$

Probiotic candidate test

To ensure the isolate's suitability as probiotic candidates, bacterial isolates were then subjected to an antibiotic susceptibility test, acid tolerance test, and non-pathogenicity test.

Antibiotic susceptibility

Isolates' sensitivity to several antibiotics was done according to previous methods (Clinical and Laboratory Standards Institute, 2015). A 24 h culture of isolates was inoculated to Marine Agar medium and then four discs of antibiotics (Oxytetracycline 30 µg, Kanamycin 30 µg, Ampicillin 10 µg and Enrofloxacin 5 µg, Reimschuessel *et al*. (2013) were placed on top of the agar. Cultures were grown overnight, and clear zones were checked.

Acid resistance

Bacterial isolate's ability to resist acidic environments in abalone stomachs was simulated with colony growth on low pH media. Marine Broth and Agar medium were adjusted to pH 4 using 1 N HCl. The isolate was then cultured for 72 h in broth before inoculating on agar and incubated for 24 h. Bacterial growth was observed as a colony presence in acidic agar medium.

Safety test

Non-pathogen properties of the isolate were examined by host injection to ensure the safety of bacteria for application in aquatic organisms. Tilapia (*Oreochromis* sp.), ±6 g each, was intraperitoneally injected with 0.1 ml of 24 h isolate culture ($1.5x10^6$ cfu/ml). A phosphate buffer solution was used to dilute isolates and as negative control). We were observed for pathogenicity signs for 7 days and mortality was recorded.

Identification of agarolytic bacteria

Bacterial isolates which displayed a stronger agarolytic index, sensitive to several antibiotics, resistant to a low pH environment and had non-pathogen properties were subjected to molecular identification and phenotypical characterization. For molecular identification, bacterial genomic DNA was extracted with Promega Wizard ® DNA Extraction Kit according to manufacturer instructions. Briefly, 24 h culture was diluted with EDTA and lysed with lysozyme 20 mg/ml and Nuclei Lysis solution. DNA was purified with RNAse A 4 mg/ml and protein precipitation solution, then precipitated using isopropanol and 70% ethanol. The 16S rRNA genes of FRAgK1 were amplified using 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-AGA GTT TGA TCC TGG CTC AG-3') Primer with the target of 1500 bp in a 50 µl volume reaction (25 µl MyTaq Red Mix, 2 µl DNA template, 2 µl primer F, 2 µl primer R, dan NFW) according to Istiqomah *et al*. (2019) protocol with 35 denaturation cycle (Primary denaturation 95 °C for 5 minutes followed by 95 °C for 30 s, annealing 55 °C for 30 s, and elongation 72 °C for 60 s. The product was then confirmed on a 1.5% gel electrophoresis and Sanger sequenced (1st BASE by PT. Genetika Science Indonesia service). Genetic similarities of the isolate were determined using BLAST (blastn) then phylogenetic trees were constructed to visualize the genetic similarity between species using the Neighbor-Joining method with *a* bootstrap value of 1000 in MEGA 11. The phenotypic examination was done by using includes Gram staining, oxidase, catalase, O/F, motility, indole production, ornithine decarboxylase, and various carbohydrate fermentation tests.

RESULTS AND DISCUSSION

Screening of agarolytic bacteria

Two pieces of Rhodophyta, identified as *Gracilaria* sp., were collected and named FRAg1 and FRAg2. Seven isolates were obtained from the sample and entire isolates showed agarolytic activity during the primary screening. Morphology of isolated bacteria (Table 1) showed most bacteria are white, circular, smooth, and small. Several unique biofilm colonies were observed. All isolates showed agarolytic activity with the presence of clear zones after Lugol's iodin spreading with the highest agarolytic index scored by FRAgK1.

This agarolytic character indicates the production of agarase by isolates to break hydrogen bonds in the polysaccharide agarose. The agarolytic index (AI) character of isolates K1 and K2 was 6.3 and 3.3, respectively. Isolate K7, K3, and K6 range from 2.1-2.4, while K4 and K5 isolates have clear zones as large as their biofilm. These results were compared with the agarolytic characters studied by Kandasamy *et al*. (2020) namely the species *Shewanella algae* and *Microbulbifer elongatus* obtained from *Kappaphycus* sp. and *Sargassum* sp.; *Vibrio natriegens* from seaweed and digestive tract of abalone in Lombok (Faturrahman, 2013) isolates N1, N2, BSUC 2, and BSUC4 from *Gracilaria salicornia* and *Gelidium latifolium* with IA ranging from 2.27 to 4.28 (Kawaroe *et al*., 2017). The agarolytic character of the isolate *Alteromonas macleodii* BC-3.1 isolated from marine sediments in Sulawesi had an index of 10 (Zilda *et al*., 2021).

Qualitative characteristics of agarase from agarolytic bacteria in an agar medium are related to the molecular mass of agarase, those characteristics are a) clearing zones after Lugol's iodine dispersion; b) visible liquefaction; dan c) depression (Vera *et al*. 1998). All isolates displayed clearing zones without liquefaction or depression, therefore it is suspected that agarase produced by all isolates has a small molecular mass. Clearing zones are caused by the inability of iodine to react with oligosaccharides resulting from polymers (repetitive structures) on agar that has been broken (Fu & Kim, 2010). The hydrolysis of agar polysaccharides on hydrogen bonds due to agarase enzymes, both α- and β-agarase. Agar that is not hydrolyzed by agarase still has the linear polysaccharide of agar (agarose) that will be coloured brown by Lugol's iodine (I_2KI, iodine-potassium iodide) as seen in Figure 1.

Table 1. Colony morphology and agarolytic index (AI) of isolates.

Isolate	Colour, Shape	Surface, Margin	Size	AI
FRAgK1	White, Circular	Rough, Margin	Small	6.3
FRAgK2	White, Circular	Smooth, Undulate	Small	3.3
FRAgK3	White, Circular	Smooth, Entire	Small	2.4
FRAgK4	White, Irregular	Smooth, Rhizoid	Large	1.0
FRAgK5	White, Irregular	Smooth, Rhizoid	Large	1.0
FRAgK6	White, Circular	Smooth, Entire	Small	2.5
FRAgK7	White, Circular	Smooth, Entire	Small	2.1

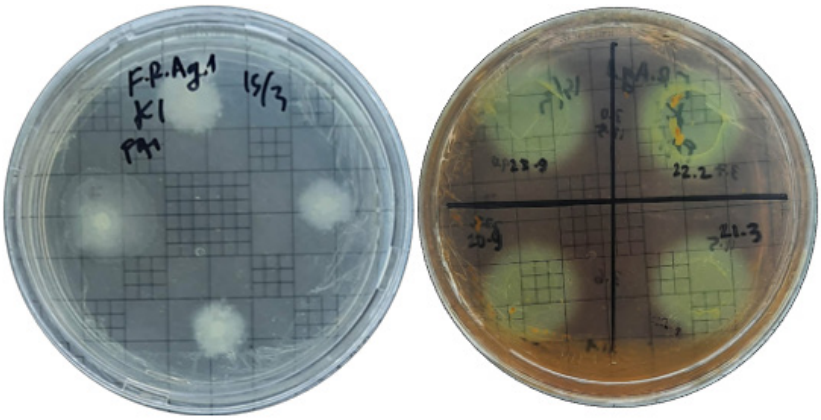

Figure 1. Colony morphology (left) and clear zones (right) were obtained by the bacteria in the agarolytic test.

Probiotic candidate evaluation

Probiotic candidates were mainly selected based on their in vitro activity. The selection criteria for probiotics include the absence of pathogenic bacteria that can harm the host, as well as the absence of antibiotic resistance or virulent genes. Probiotics must reach their intended destination, namely the digestive system, intact by being resistant to acid pH fluctuations, bile acids, strong adhesion, and positive competition against intestinal bacterial colonies. Probiotic bacteria must primarily produce digestive enzymes and be resistant to infections. (Wuertz *et al*., 2021). In the present study, strain FRAgK6 and FRAgK7 did not exhibit adequate acid resistance, but the remaining isolates exhibited favorable growth on acidic agar as evidenced by colony presence. In antibiotic susceptibility tests, most bacteria were sensitive to commercially available oxytetracycline (30 ug), whereas most resistance was observed on low concentrations of enrofloxacin (5 ug) and ampicillin (10 ug), indicating that higher concentrations were required to observe bacterial sensitivity. Intermediate susceptibilities were determined by the formation of hazy zones between the isolate and the paper disc. The FRAgK1 strain in the present study was susceptible to oxytetracycline, kanamycin, and ampicillin. This result was similar to the previously described Bacillus sp. (AlGburri *et al*., 2016). Pathogenic features were discovered on FRAgK4 and FRAgK5, which resulted in 100% mortality in the Oreochromis sp. host, whereas the remaining isolates were non-pathogenic with 0% mortality. Isolate FRAgK1 has been chosen as a probiotic's candidate because of its exceptional qualities, such as agarolytic ability, acid tolerance, nonpathogenicity, and sensitivity to tested antibiotics (Table 2).

Table 2. Low pH resistance, antibiotic susceptibility and safety of the bacteria in the present study.

Isolate	Growth on pH 4 condition	Antibiotic susceptibility				Fish mortality due to bacterial injection (%)
		Otc	Knm	Enf	Amp	
FRAgK1	+	S	S	S	S	0
FRAgK2	+	S	I	I	S	0
FRAgK3	+	S	I	I	R	0
FRAgK4	+	S	R	R	R	100
FRAgK5	+	S	S	R	I	100
FRAgK6	–	S	I	S	R	0
FRAgK7	–	S	I	S	R	0

Otc: oxytetracycline, Knm: kanamycin, Enf: Enrofloxacin, Amp: Ampicillin, S: sensitive, I: intermediate sensitive, R: resistant

Identification of agarolytic bacterium

A partial sequence of the 16S rRNA gene of FRAgK1 was obtained with a length of 1234 bp and was sufficient for BLAST comparison (Table 3). Several known agarolytic bacteria accession numbers were recovered from various publications. The isolate was revealed to have 99.43% similarity over *B. subtilis* strain DSM 10. Genetic similarities of FRAgK1 were presented by a phylogenetic tree in Figure 2 with known agarolytic bacteria and most related species. A 99.5% or higher threshold of the 16S rRNA gene sequence is used to include a bacterium as a strain of species, thus the isolate is named *Bacillus* sp. FRAgK1. Further molecular identification by using multilocus analysis is required to confirm the bacterial identification to species level.

Bacillus subtilis is a member of the phylum Firmicutes, Class Bacilli, Order Bacillales, Family Bacillaceae, and Genus Bacillus (Elshaghabee *et al*., 2017). The bacterium has become a model of the genus Bacillus which is highly researched and capable of making biofilms. *B subtilis* is found in various habitats, including land (terrestrial), plant surfaces, animal digestion, fresh waters, to marine waters (Earl *et al*., 2008). The genus Bacillus is generally important bacteria in environment (Borriss *et al*., 2020), a potential probiotics candidate (Andriani *et al*., 2017) that had been isolated from the gut of various freshwater and marine organisms, e.g.: tilapia, carp fish, shrimp, and abalone (Nayak, 2020).

Bacillus subtilis can secrete a variety of enzymes which make this species often used in industry and probiotics. Latorre *et al*. (2016) described the four types of enzymes produced naturally by these bacteria, namely: α-amylase, protease, lipase, and phytase. *B. subtilis* has been observed to have an agarolytic character and produce extracellular agarase which is stable to environmental conditions with the highest enzyme activity value of 2.3 U per litre (Saraswathi *et al*., 2011). The use of *Bacillus subtilis* in aquaculture has been investigated as a probiotic, bioremediation, synbiotic, and biocontrol agent (Nayak, 2020). In the field of probiotics, *B. subtilis* has been observed to be capable of producing digestive enzymes, antimicrobial substances and exopolymeric substances, increasing the synergy between the gastrointestinal bacterial community; and competing with pathogenic bacteria.

Table 3. BLAST similarity of *Bacillus* sp. FRAgK1 isolate to references.

Species	Query cov. (%)	Per. Ident. (%)	Accession number [Ref.]
Most similarity			
Bacillus subtilis strain DSM 10	99	99.43	NR027552.1
Bacillus subtilis strain BCRC 10255	99	99.43	NR116017.1
Bacillus inaquosorum strain BGSC	99	99.35	NR104873.1
Bacillus subtilis subsp. subtilis strain 168	99	99.27	NR102783.2
Bacillus spizizenii strain NRRL B-23049	99	99.27	NR024931.1
Known agarolytic bacteria			
Bacillus sp. strain MK03	98	94.62	AB062678.1 (Suzuki et al., 2003)
Microbulbifer sp. strain SD-1	81	82.47	(Kim et al., 2011)
Microbulbifer elongatus strain PORT2	81	82.50	MH622756.1 (Anggraeni &Ansorge-Schumacher, 2021)
Alteromonas macleodii strain BC31	81	82.51	MT325874.1 (Zilda et al., 2021)
Microbulbifer elongatus strain JAMB A7	81	82.39	AB107975.1 (Ohta et al., 2004)
Alteromonas macleodii GNUM-08120	81	82.29	JN578475.1 (Chi et al., 2012)
Microbacterium sp. strain SELA4	83	81.69	MG203882.1 (Parashar & Kumar, 2018)
Streptomyces coelicolor A3(2)	84	81.00	NC003888.3 (Temuujin et al., 2011)
Vibrio astriarenae strain HN897	81	81.26	CP047475.1 (Liu et al., 2020)
Vibrio sp. strain V134	81	81.04	EF100710.1 (Zhang & Sun, 2007)
Thalassomonas agarivorans JAMB A33	83	80.12	AB162002.1 (Ohta et al., 2005)
Pseudoalteromonas sp. CKT1	82	79.88	AB036070.2 (Chiura & Kita-Tsukamoto, 2000)
Agarivorans sp. LQ48	82	79.67	FJ593496.1 (Long et al., 2009)
Pseudoalteromonas antarctica strain N1	84	78.90	AF045560.1 (Vera et al., 1998)
Agarivorans albus strain YKW-34	63	80.41	EU084496.1 (Fu et al., 2008)
Flammeovirga yaeyamensis strain MY04	81	77.04	AY849869.1 (Han et al., 2012)
Flammeovirga yaeyamensis strain YT	81	77.04	(Yang et al., 2011)
Tamlana sp. Aga22	81	75.54	AM946640.1 (Zhao et al., 2009)

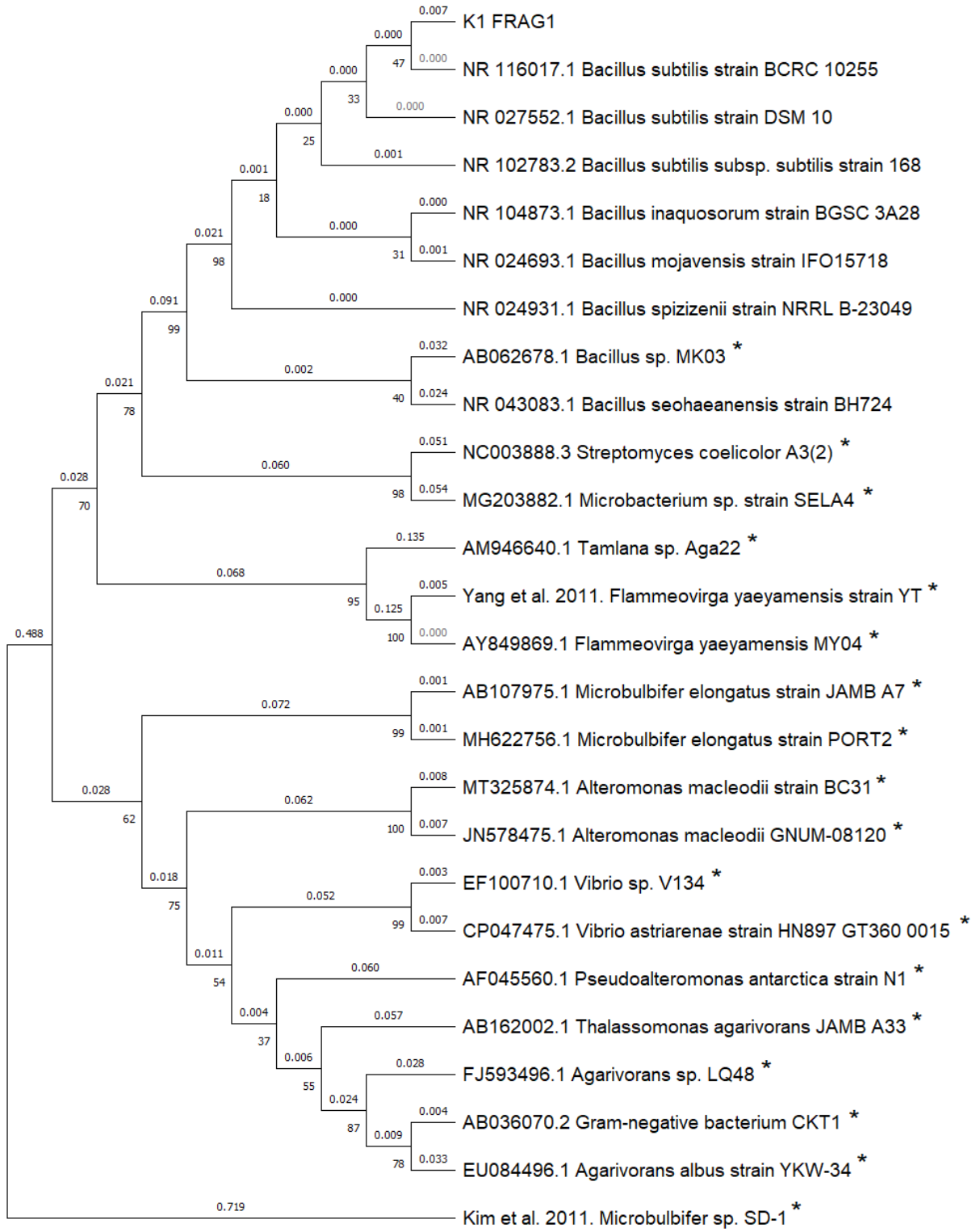

Figure 2. Phylogenetic tree of FRAgK1 strain with highly similar strains and known agarolytic bacteria (*).

Phenotypic characterization found that the FRAgK1 was a short-rod Gram-positive bacteria that produces oxidase, ornithine decarboxylase and use glucose as a source of carbon (Table 4). The bacterium did not motile, did not produce catalase and indol, and could not ferment lactose and sucrose. The non-motile character supporting to the suggestion that the present bacterium might be new species of Bacillus because it is different to the character of *B. subtilis* (Logan & Vos, 2015).

A further study to determine quantitatively agarase production and optimum conditions for *Bacillus* sp. FRAgK1 need to be done. An in vivo test to confirm the colonization ability of the FRAgK1 on *Gracilaria* sp. thalli (Rosenberg *et al*., 2016) by metagenomic analysis and the bacterial ability to digest the agar contents is interesting to confirm. Moreover, further characterization based on the other criteria for aquaculture probiotics is also required. The present bacteria might be a good candidate for probiotics to support the red seaweed digestion process by the abalone stomach to enhance the growth rate in aquaculture.

Table 4. Phenotypic characteristics of FRAgK1 are isolated with reference.

Phenotype	FRagK1	Bacillus subtilis (Logan & Vos, 2015)
Cell morphology	Short rod	rod
Gram	+	+
Motility	–	+
Oxidase	+	d
Catalase	–	+
Indole	–	–
Ornithine decarboxylase	+	–
Glucose	+	+
Lactose	–	–
Sucrose	–	+

d: dependable on the strain.

CONCLUSION AND RECOMMENDATION

Conclusion

An agarolytic bacterium, *Bacillus* sp. FRAgK1 obtained from red seaweed *Gracilaria* sp. is a potential gut probiotic for abalone with an agarolytic index of 6.3, acid resistance, antibiotic sensitivity, and non-pathogenic features.

Recommendation

Further investigation into the probiotic application trial in abalone culture is essential.

AUTHORS' CONTRIBUTIONS

FZA conducted the research and wrote the manuscript; II design the study, analysed the data, and wrote the manuscript; AI examined the data and reviewed the manuscript; NSY designed the study and reviewed the manuscript; NI examined the data and reviewed the manuscript.

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