Bioprospecting For Bacterial Endophytes Associated With Zingiberaceae Family Rhizomes In Sibolangit Forest, North Sumatera

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Abstract
The present study was conducted aiming to isolate and characterize endophytic bacterial isolates with antibacterial ability, phosphate solubilization, and proteolytic activity from rhizomes of the Zingiberaceae family (Etlingera sp., Globba patens, Globba pendula, and Zingiber multibracteata). Nineteen bacterial isolates were obtained from Zingiberaceae rhizomes with isolate codes of EZS27, EZS18, EZS19, EZS25, EZS16, EZS08, EZS09, EZS13, EZS20, EZS14, EZS10, EZS11, EZS03, EZS05, EZS06, EZS43, EZS45, EZS47, and EZS28. The screening of the endophytes for antibacterial activity was done through the paper disc method. Four bacterial isolates presented antibacterial activities. EZS06 isolate inhibited the growth of EPEC (11 mm), P. vulgaris ATCC 13315 (10 mm), and L. monocytogenes BTCC B693 (9 mm). Also, EZS20 isolate inhibited the growth of S. aureus ATCC 29213 (17 mm), EZS28 isolate inhibited MRSA ATCC 43300 (8.6 mm), and EZS45 isolate inhibited S. Epidermidis ATCC 12228 (9 mm). The EZS19, EZS03, and EZS16 isolates dissolved the phosphate most effectively. Eight isolates (EZS19, EZS47, EZS27, EZS25, EZS09, EZS20, EZS45, and EZS06) showed the best protease activity. In general, our results showed that the endophytic bacterial strains can be used as a new and useful antibacterial agent since it showed antibacterial activity and chemical diversity. Furthermore, it also has the potential for exploitation in a wide variety of medical, agricultural, and industrial areas.

Keywords: *Endophytic bacteria, antibacterial, phosphate solubilization, proteolytic, zingiberaceae.*

I. INTRODUCTION

Endophytes can be considered as a diverse microorganism community including archaea, actinobacteria, fungi, and bacteria thatpresent a symbiotic relationship with the plant tissue and play an

important role in plant growth, defense against plant disease, and diversification. The endophyte diversity is significantly determined by plants' environmental factors. For further explanation,Maheshwari*et al* [1] stated that the endophyte diversity up to the genotype level is affected by the plants'environment and species. There are about 300,000 plant species living on Earth, and each of them is the host for one or more endophytic [2]. There is mutualism symbiotic relationship between the endophytes and its host plant. It is generally known that endophytic bacteria can induct protection for its host by producing siderophoresand presenting metabolite activities such as antifungal[3,4]. There are several endophytic bacteria that also affect plants' growth stimulation and nitrogen fixation [5,6].

Therefore, this study on endophytic bacteria can help to know the most effective role and potential in its application. The endophytic bacteria provides several bioactive compounds consisting of various types of secondary metabolite [7]. Bioactive metabolite has been widely applied as antimicrobial, immunosuppressant, antiparasitic, antioxidant, anticancer, and antidiabetic [8,9]. Many researchers have reported that endophyte isolated from medicinal plants is very good to be used as fungicide, bactericide, and cytotoxic metabolites [10].Considering the importance of the endophytes, the researchers were encouragedto review and study the potential of endophytic bacteria isolates from the plants of the Zingiberaceae family collected from Sibolangit forest, North Sumatera in the context of bioprospecting that further can be used for application need in the future.

II. METHOD

2.1. Samples collection

The Zingiberaceae rhizomes were collected carefully from four species ofthe Zingiberaceaefamily, consisting of *Etlingera* sp., *Globba patens*, *Globba pendula*, and *Zingiber multibracteata* locaten in Sibolangit forest (3^0 17 50 -3^0 18 39 LU and 98^0 36 0 -98^0 36'36 BT), North Sumatera Indonesia(Fig. 1).

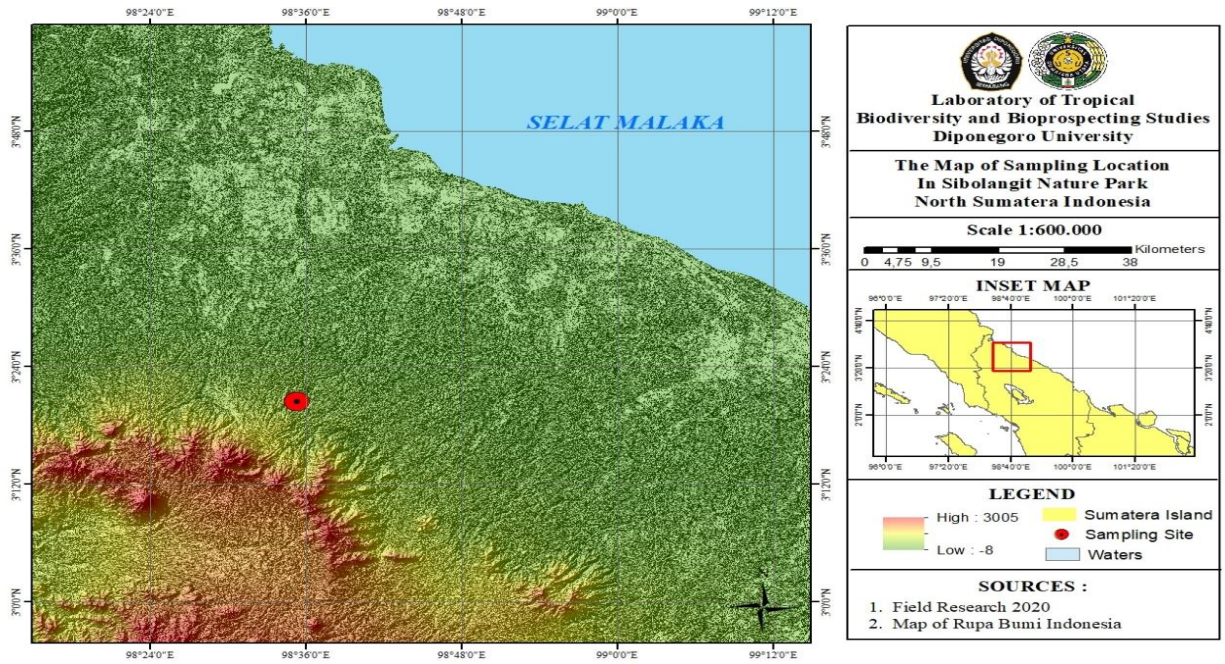

Fig. 1. Sample collection sites in Sibolangit forest, North Sumatera province, Indonesia

2.2. Endophytic Bacterial Isolates from Zingiberaceae Rhizomes

This descriptive study isolated and identified the endophytic bacteria and characterized its activity by using the Kirby-Bauer method on EPEC, *S. aureus* ATCC 29213, *L. monocytogenes* BTCC B693, MRSA ATCC 43300, *S. epidermidis* ATCC 12228 and *P. vulgaris* ATCC 13315. Surface sterilization was done in this research to isolate the endophytic bacteria from Zingiberaceae rhizomes. In the surface sterilization process, the samples were first washed using running water and then continued by the combination of water and sunlight [11]. After being brushed, small pieces in the size of 1-2 cm were obtainedby cutting the root surface. Then, the pieces were immersed into various media as follow:3 minutes in alcohol 70%,then washed with distilled water;5 minutes in1% sodium hypochlorite and washed again in sterile distilled water; and then 1 minute in 70% alcohol and back to sterile distilled water media again for 2 minutes. After the pieces were soaked in various washing steps, they were then placed on the surface of the mix of solid "Nutrient Agar (NA)" media mixed and ketocenazole antibiotics (0.3 g/100 ml) in which the cut part was positioned to be attached towards the media. Then, they were incubated for 3 days at 37°C to the growth of the colonies. The colonies were then observed through purification in a new NA media.

2.3. Morphological and Biochemical Characteristic of the Endophytic Bacteria

The morphological characterization of the endophytic bacteria was done byusing the macroscopic method. The first morphological characteristic identified was the shape of the colony formed. The isolates of the endophytic bacteria were then identified through the reaction of traditional Gram stain [12] and observed using a compound bright-field microscope (OLYMPUS CH20BIMF200) with 100x magnification. The endophytic bacteria were also tested regarding its biochemical profile includingstarch hydrolysis test, gelatin hydrolysis test, Simon citrate agar, hydrogen sulfide, motility, and catalase test.

2.4. The Antagonistic Test of Endophytic Bacterial Isolate against Pathogenic Bacteria

The pathogenic bacteria employed in this research included the entero pathogenic of *Escherichia coli* (EPEC), *Staphylococcus aureus* ATCC 29213, *Listeria monocytogenes* BTCC B693, *Methicillin-resistant Staphylococcus aureus* (MRSA) ATCC 43300, *Staphylococcus epidermidis* ATCC 12228, and *Proteus vulgaris* ATCC 13315. For the tests, the selected strains were rejuvenated in 5 mL of NB media and incubated at 37°C in shaker incubator for overnight for the antagonistic test of endophytic bacterial isolates. Sterile NaCl of 0.85% was added into the bacterial cultures in order to dilute its initial volume by 9 times. As much as 17 mL of MHA (Mueller Hinton Agar) media was prepared and added by 3 mL of the dilution result in which then the mix was homogenized. The combination of the bacteria and media was placed into a petri dish until it became solidified. Paper discs were arranged on media that was already doped with 30 µL of endophytic bacteria. Incubation was done on the samples at 37°C for 24 hours. The measurement of the diameter of the clear zone formedafter the incubation was conducted todetermineits antimicrobial activity values.

2.5. Screening of the Endophytic Bacteria against Phosphate Solvent Media

Solid Pikovskaya medium [13] was modified regarding its composition (g/L) of glucose (10.0), $(NH_4)_2$ SO_4 (0.5), Ca_3 $(PO_4)_2$ (5.0), NaCl (0.3), $MgSO_4$.$7H_20$ (0.3), KCl (0.2), $MnSO_4.4H_2O$ (0.03), $FeSO_4.7H_2O$ (0.03), Yeast extract (0.5), agar (15.0), and equates (1000 mL) as a media to grow the endophytic bacteria. The endophytic bacteria colonies that dissolve the phosphate will create a spectrum wide observed by growing the bacteria using zig-zag method. Incubation is the last process conducted for 24 hours at a temperature of 25˚C to observe the bacteria's phosphate dissolving ability. Bacteria thatsuccessfully dissolved phosphate created a clear zone around the colony.

III. RESULT AND DISCUSSION

3.1. Isolation, physiology, and biochemical characteristics of endophytic bacteria In total 19 endophytic bacterial isolates were obtained and purified from the rhizome of the Zingiberaceae (*Etlingera* sp.,

Globba patens, *Globba pendula*, and*Zingiber multibracteata*). There were a total of 11 isolated from the endophytic bacteria of *Globba patens*, identified asEZS18, EZS19, EZS25, EZS16, EZS09, EZS20, EZS14, EZS10, EZS11, EZS03, and EZS13. Four isolates were successfully obtained from *Globba* pendula, identified asEZS05, EZS06, EZS08, and EZS43. Furthermore, *Zingiber multibracteata*successfully produced 3 bacterial isolates including EZS45, EZS47, and EZS28. Meanwhile, *Etlingera* sp. obtained 1 endophytic bacterial isolate namelyEZS27. Based on the morphology of colony and gram coloring, the endophytic bacteria of zingiberaceae rhizome (Tab. 1) shows that all isolates come from zingiberaceae rhizome by having two shape-types of cells, which are cocci and bacilli. The most frequently found shape of the endophytic bacteria was cocci, which accounted for 16 isolates.

Tab. 1. Shape and Gram Characteristic of Endophytic Bacterial Isolate from Zingiberaceae

Isolate Code	Cell Shape	Gram
EZS27	Cocci	Negative
EZS18	Cocci	Positive
EZS19	Cocci	Negative
EZS25	Cocci	Negative
EZS16	Cocci	Negative
EZS08	Bacilli	Positive
EZS09	Cocci	Negative
EZS13	Bacilli	Positive
EZS20	Bacilli	Positive
EZS14	Cocci	Negative
EZS10	Cocci	Negative
EZS11	Cocci	Negative
EZS03	Cocci	Negative
EZS05	Cocci	Negative
EZS06	Cocci	Negative
EZS43	Cocci	Negative
EZS45	Cocci	Negative
EZS47	Cocci	Negative
EZS28	Cocci	Negative

The endophytic bacterial isolates obtained in this research were mostly dominated by negative gram bacteria types except for EZS18, EZS08, EZ013, and EZS20, which werepositive gram. The major phylum of gram-negative bacteria is Proteobacteria,which is a group most frequently found in plants' tissue and mostly are endophytic bacteria isolated from various plants [14-16]. Previous studies isolated endophytic bacteria from the Zingiberaceaefamily and identified as*Alpinia galanga*[17-19], *Stahlianthus campanulatus* [20], and*Zingiber montanum* [21].However, studies for the isolation and characterization of endophytic bacteria of Zingiberaceae Rhizomes from Sibolangit Forest, North Sumatera are rare. Therefore, this is the first report of the isolation and characterization of endophytic bacteria from Zingiberaceae family speciesthat are*Etlingera* sp., *Globba patens*, *Globba pendula*, and *Zingiber multibracteata*.

Table 2 shows that all isolates from the Sibolangit forest have catalase activity (Fig. 1). Catalase is an important enzyme for bacteria cells and usually presents in bacteria cells through aerobic metabolism [22].In this research, it was found that 16bacterial isolates presented motility, and three were non-motile. There were 4 isolates thatcan hydrolyze starch, which means that carbohydrate is a carbon source for bacteria growth nutrition.

Tab. 2. Physiology and Biochemical Characteristics of Endophytic Bacterial Isolates of Zingiberaceae

Isolate Code	Starch Hydrolysis	Gelatin Hydrolysis	Citrate Test	Hydrogen Sulfide	Motility	Catalase Test	Triple Sugar Test	
							Deep	Slant
EZS27	+	+	-	-	+	+	Yellow	Red
EZS18	-	-	+	-	+	+	Yellow	Yellow
EZS19	-	+	+	-	-	+	Red	Red
EZS25	-	-	+	-	-	+	Red	Red
EZS16	-	+	+	-	-	+	Red	Red
EZS08	+	-	+	-	+	+	Red	Yellow
EZS09	+	+	+	-	+	+	Yellow	Red
EZS13	-	-	-	-	+	+	Yellow	Yellow
EZS20	+	-	-	-	+	+	Yellow	Yellow
EZS14	-	-	+	-	+	+	Yellow	Yellow
EZS10	-	-	+	-	+	+	Yellow	Yellow
EZS11	-	-	+	-	+	+	Yellow	Yellow
EZS03	-	-	-	-	-	+	Red	Red
EZS05	-	-	+	-	+	+	Yellow	Yellow
EZS06	-	-	+	-	-	+	Red	Red
EZS43	-	-	+	-	+	+	Red	Red
EZS45	-	-	+	-	+	+	Yellow	Yellow
EZS47	-	+	+	-	+	+	Yellow	Red
EZS28	-	+	+	-	+	+	Yellow	Yellow

After bacterial culture examination, we conducted a motility test and found that they were motile (Fig.2). Sixteen bacterial isolates that had motile properties are EZS27, EZS18, EZS08, EZS09, EZS13, EZS20, EZS14, EZS10, EZS11, EZS05, EZS43, EZS45, EZS47, and EZS28. Meanwhile, the motility test of the other three isolates, namelyEZS19, EZS25, EZS16, EZS03, and EZS06,showed negative. Motility is the bacteria's ability to move along in the growth medium.

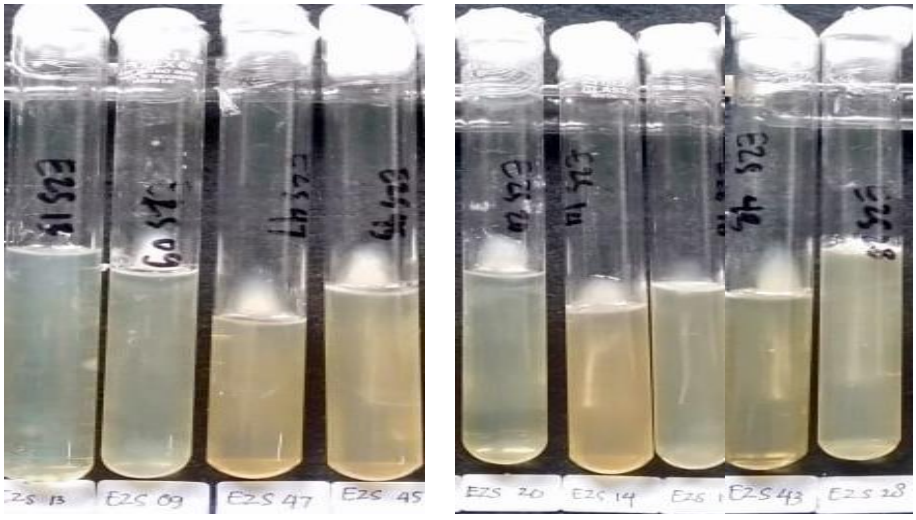

Fig. 2. Result of motility property test of endophytic bacteria from zingiberaceae rhizomes

Based on the research result on TSIA (Triple Sugar Iron Agar) media (Fig. 3), the reaction that can be seen is that all parts of the media which are the slant and butt shows red color indicating that the media was base (alkali).

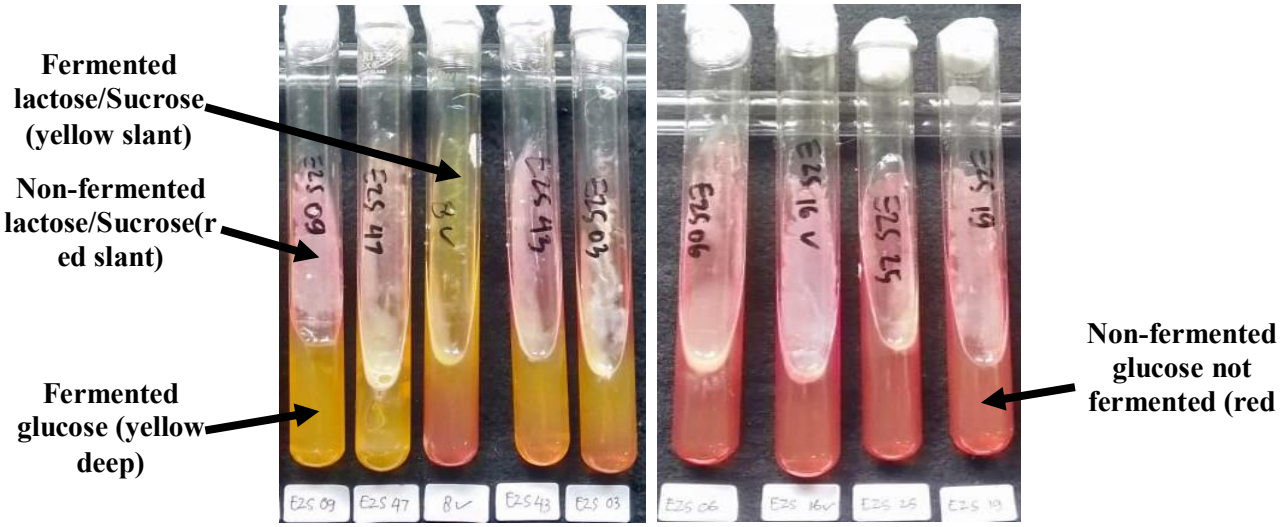

Fig. 3. The interpretation of TSIA test result of endophytic bacteria from zingiberaceae rhizomes

Theendophytic bacteria from zingiberaceae rhizomesin this research were mostly obtained by using citrate as carbon and energy source except for EZS27, EZS13, EZS20, and EZS03 isolates, which did not use citrate as the sole carbon and energy source. The endophytic bacteria that used citrate as a carbon source producedsodium carbonate that is alkali so that the indicator of bromothymol blue caused the media to be blue (Fig. 4). If the endophytic bacteria is able to use citrate, then the acid will be removed from the growth media and causing the increase of pH which eventually changes the medium color from green into blue.

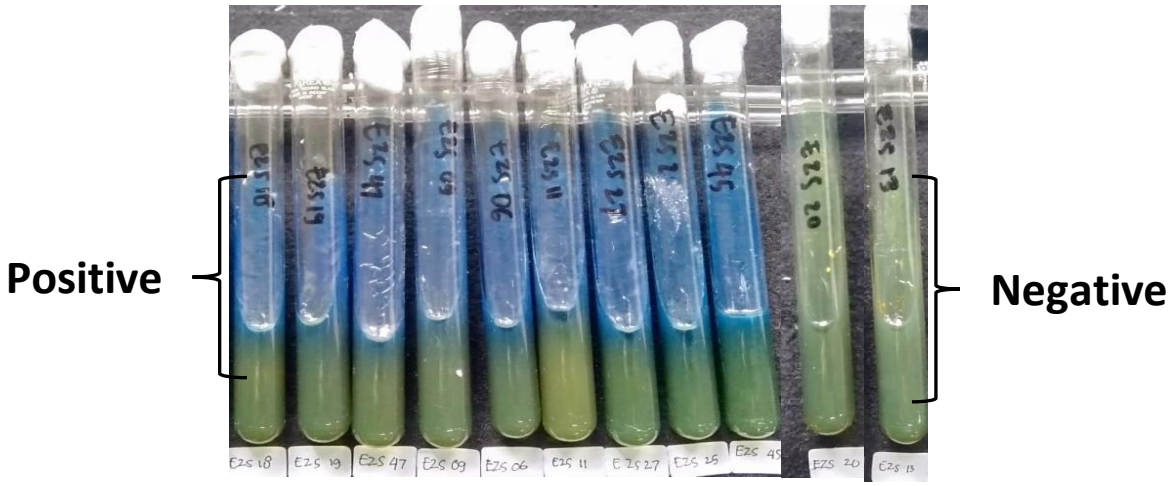

Fig. 4. Test result of endophytic bacteria from zingiberaceae rhizomes onSimon'sCitrate Agar

3.2. Antibacterial activity

Few isolates showed antibacterial activity indicated bya clear zone of inhibition. There were 5 isolates that showed a broad spectrum of contrasting antibacterial activity by forming the highest zone of inhibition observed against all pathogenic bacteria test. The endophytic bacteria showed antibacterial activity against selected pathogenic strains as shown in Tab. 3.

Tab. 3. Screening for Antibacterial potential

Bacterial isolate codes	**Antibacterial activity (mm)**					
	EPEC	*S. aureus* ATCC 29213	*L. monocytogenes* BTCC B693	MRSA ATCC 43300	*S. epidermidis* ATCC 12228	*P. vulgaris* ATCC 13315
EZS27	6.8	-	-	7	6.5	7
EZS18	7	9	-	6.5	-	7
EZS19	7	6.6	-	7	-	6.8
EZS25	7	-	-	6.8	-	7
EZS16	-	-	-	-	-	6.8
EZS08	-	9	6.6	7	-	7
EZS09	7	-	7	6.8	-	7
EZS13	-	-	-	-	7	6.6
EZS20	-	**17**	-	-	7	-
EZS14	6.8	-	-	7	6.6	7
EZS10	7	8.8	-	-	-	7
EZS11	9	-	-	-	7	6.8
EZS03	9	-	-	-	-	8.6
EZS05	7	9	-	-	8.6	7
EZS06	**11**	7	**9**	-	7	**10**
EZS43	7	6.6	7	-	-	6.5
EZS45	6.5	7	7	6.8	**9**	7
EZS47	7	9	6.8	7	6.5	-
EZS28	7	-	-	**8.6**	-	6.8

Note:*Clear zone was measured after incubation for 24 h ± 28°C to identify the activity. Bolded number is the biggest clear zone of inhibition.*

The antibacterial activityof the endophytic bacteria is presented in Fig. 5. The arrow shows the best antibacterial activity from the endophytic bacterial isolate fromzingiberaceae. Many researchers found new drugs from endophytic bacteria to control human disease due to its antibacterial activities. For this research, the endophytic bacteria were tested using 6 pathogenic bacteria that are related to the human body. Most of the endophytic bacteria are sensitive to test pathogenic bacteria but some of them are also resistant. The antibacterial activities fromendophytes of the zingiberaceae family have been reported by several researchers. *Stenotrophomonas maltophilia*, *Bacillus safensis*, *Bacillus pumilus*, and*Brevibacterium halotolerans*were successfully isolated from *Curcuma longa* [22].

This research successfully obtained the isolate EZS06 from *Globba pendula* rhizomes which has the best activity in inhibiting EPEC growth (11 mm), *P. vulgaris* ATCC 13315 (10 mm),and*L. monocytogenes* BTCC B693 (9 mm), EZS20 isolate from*Globba patens* inhibits*S. aureus* ATCC 29213 (17 mm), EZS28 isolate from*Zingiber multibracteata*inhibits MRSA ATCC 43300 (8.6 mm), and EZS45 isolate from*Zingiber multibracteata*inhibits*S. epidermidis* ATCC 12228 (9 mm). The result of the phytopharmacological study found that many Zingiberaceae family species have various secondary metabolites containing a great potential to be applied in pharmaceutical field, including as an antioxidant,

anti-inflammatory, antimutagenic,antibacterial, antidiabetic, expectorant, hepatoprotective, and anticancer properties [23].

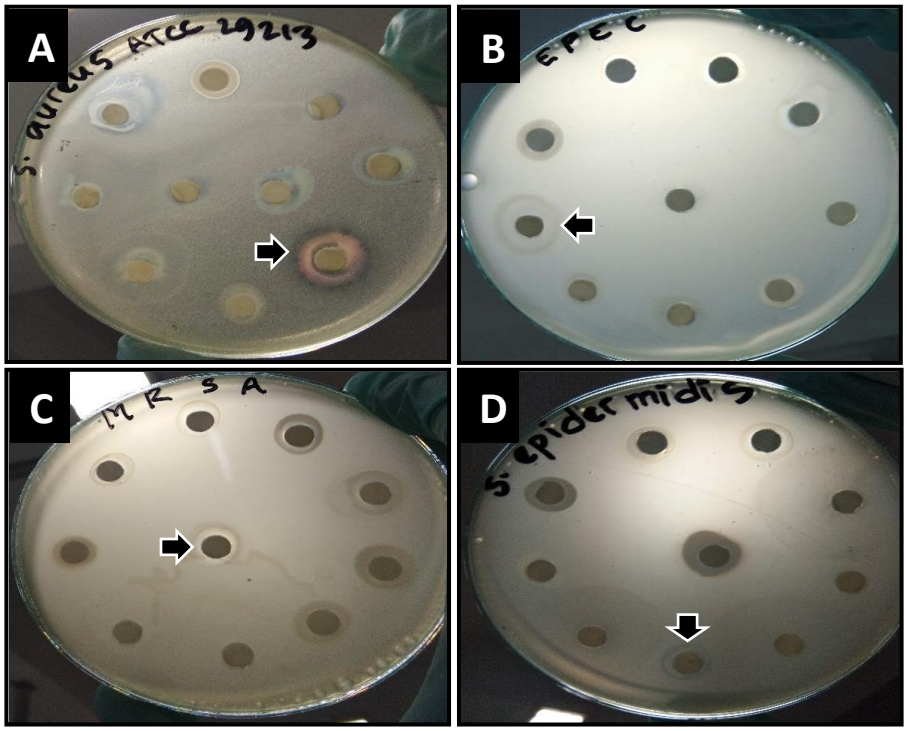

Fig. 5. The inhibition zone formed during antibacterial activity by endophytic bacteria; A. EZS20 isolate towards*S. aureus* ATCC 29213, B. EZS06 isolate towards EPEC (Enteropathogenic *E. coli*), C. EZS28 isolate towards MRSA ATCC 43300, D. EZS45 isolate towards*S. epidermidis* ATCC 12228.

3.3. PhosphateSolubilization Capability

Three endophytic bacterial isolates presented the best ability to dissolve phosphate (++), while the other two isolates can dissolve phosphate but witha low ability (+). Ten isolates did not present the ability to dissolve phosphate. The ability of endophytic bacterial isolate from zingiberaceae can be seen in Tab. 4.

Tab. 4. Screening for P-Solubilization Ability and proteolytic potential

No.	Bacterial isolate codes	Phosphate solubilization	Protease production
1	EZS19	++	++
2	EZS03	++	-
3	EZS16	++	-
4	EZS11	+	-
5	EZS47	+	++
6	EZS27	-	++
7	EZS25	-	++
8	EZS09	-	++
9	EZS08	-	+
10	EZS20	-	++
11	EZS45	-	++

12	EZS28	-	+
13	EZS06	-	++

Incubation for 24 hours at ± 28°C was performed to measure the clear zone aiming to identify the clear zone: -, no activity; +,low; ++,high.

The EZS19, EZS03, and EZS16 showed the best activity in dissolving phosphate (Fig. 6). The endophytic bacteria are inoculantswhich means that theyhave the ability to convertthe insoluble forms of soil P into accessible forms to enhance host P uptake [24]. Another factor affecting the bacteria solubilization is the P source [25-26]. It has been found that P is mandatory for plant growth and its deficiency limits plant development. Even though chemical fertilizers are added to the soils, plants can only use low phosphatic fertilizer quantity because of the immobilization of P. This result indicates that the three endophytic bacteria isolates from zingiberaceae (EZS19, EZS03, and EZS16) have the potential as plant fertilizer.

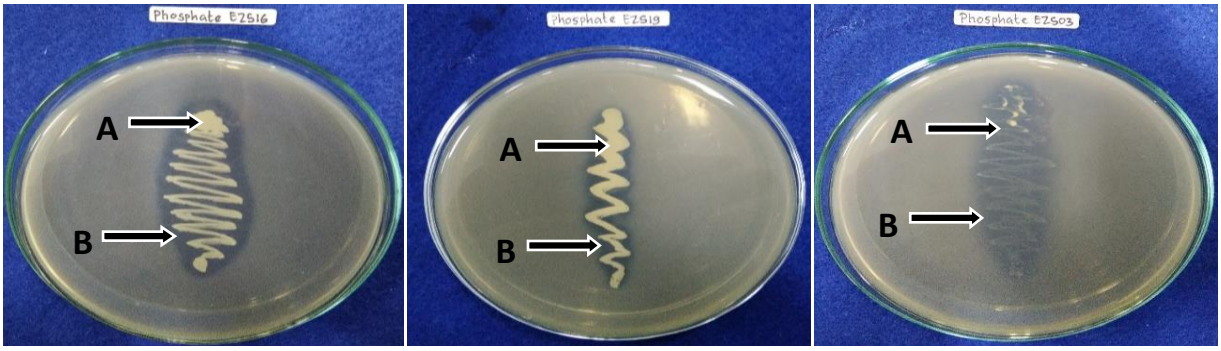

Fig. 6. Phosphate solvent endophytic bacteria in Pikovskaya media after being incubated for 24 hours. (A) Bacterial cell colonies, (B) phosphate solubilization indicated by clear zone

3.4. ProteolyticCapability

Skimmed milk agar was used to hydrolyze the protein in order to determine the presence of proteolytic enzyme. Clear zone formed around the colonies indicated that there was hydrolytic activities (Fig. 7). Ten isolates showed activities associated with proteases, eight isolates showed the best protease activity (Tab. 4). Fig. 3 shows the ability of endophytic bacteria to hydrolyze protein from skim milk. Meanwhile, the translucent zone formed around the colonies indicated the presence of substrate degradation by proteases.

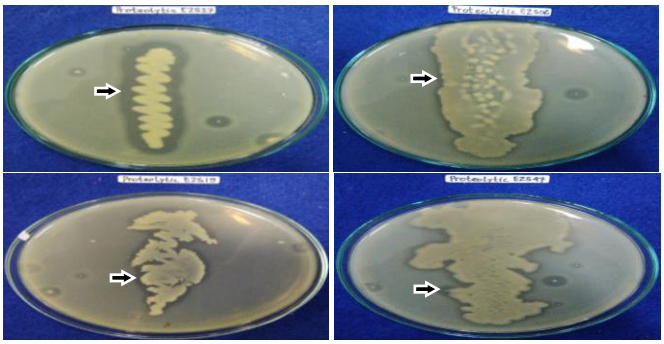

Fig. 7. Photomicrograph shows the proteolytic activity of the endophytic bacterial isolates on skim milk supplemented agar bacteriological (oxoid). The arrows indicate the proteolytic activities with clear zone around the colonies.

IV. CONCLUSION

In our study, we obtained nineteen endophytic bacterial isolates were from the zingiberaceae rhizomes. There were four bacteria isolates that have antibacterial activities. EZS06 isolate fromthe rhizome of *Globba pendula* inhibits the growth of EPEC (11 mm), *P. vulgaris* ATCC 13315 (10 mm) and*L. monocytogenes* BTCC B693 (9 mm), EZS20 isolate from *Globba patens* rhizome inhibits *S. aureus* ATCC 29213 (17 mm), EZS28 isolate from *Zingiber multibracteata*rhizome inhibits MRSA ATCC 43300 (8.6 mm), and EZS45 isolate from*Zingiber multibracteata*rhizome inhibits *S. epidermidis* ATCC 12228 (9 mm).EZS19, EZS03, and EZS16 isolates indicated the best activities of dissolving phosphate. Eight isolates (EZS19, EZS47, EZS27, EZS25, EZS09, EZS20, EZS45, and EZS06) showed the best protease activity. Endophytic bacterial from zingiberaceae rhizomes are a very promising source to produce bioactive compounds. However, it is rarely investigated although it has abundant amount. As a bioactive and chemical novel compound, it is a dependable source that has the potential to be widely applied in the medical, agricultural, and industrial fields.

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